Production of Bioactive, Post-Translationally Modified, Heterotrimeric, Human Recombinant Type-I Collagen in Transgenic Tobacco†

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Collagen's biocompatibility, biodegradability and low immunogenicity render it advantageous for extensive application in pharmaceutical or biotechnological disciplines. However, typical collagen extraction from animal or cadaver sources harbors risks including allergenicity and potential sample contamination with pathogens. In this work, two human genes encoding recombinant heterotrimeric collagen type I (rhCOL1) were successfully coexpressed in tobacco plants with the human prolyl-4-hydroxylase (P4H) and lysyl hydroxylase 3 (LH3) enzymes, responsible for key posttranslational modifications of collagen. Plants coexpressing all five vacuole-targeted proteins generated intact procollagen yields of ~2% of the extracted total soluble proteins. Plant-extracted rhCOL1 formed thermally stable triple helical structures and demonstrated biofunctionality similar to human tissue-derived collagen supporting binding and proliferation of adult peripheral blood-derived endothelial progenitor-like cells. Through a simple, safe and scalable method of rhCOL1 production and purification from tobacco plants, this work broadens the potential applications of human recombinant collagen in regenerative medicine.

Introduction

Collagens are the main structural proteins in vertebrates and many other multicellular organisms. The collagen molecules assemble to fibrils that organize to form fibers, providing unmatched structural integrity for the extracellular matrix.[1] Collagen plays additional biological roles through its interactions with a variety of macromolecules including integrins, decorin, fibronectin, heparin, and MMPs.[2]

Collagen and collagen-derived fragments control many cellular functions related to wound healing including cell shape, differentiation, migration, and protein synthesis. Thus, many collagen formulations have been successfully applied in tissue regeneration protocols and in design of medical devices.[3,4]

Type I collagen represents the prototype of fibrillar collagens, constitutes the predominant collagen in bone and tendon, and is found in significant quantities in skin, aorta, and lung. The type I collagen heterotrimer is composed of two alpha 1 and one alpha 2 chains, constructed from repeating Gly-X-Y triplets, where X and Y can represent any amino acid but are typically proline and hydroxyproline.[5] The three constituent polypeptide chains assemble to form a procollagen molecule within the rough endoplasmic reticulum (ER) assisted by the globular C-terminal extension propeptides, forming a trimeric molecule. The complex then folds in a C-to-N direction to yield a triple helix.

The complex biosynthesis of procollagen involves a number of co- and post-translational modifications, including proline and lysine hydroxylation and both intra- and interchain disulfide bond formation, all essential for the physiological stability of the final triple helix conformation. The enzymes responsible for these modifications act in a coordinated fashion to ensure appropriate folding and assembly of a correctly aligned and thermally stable triple-helical molecule.

Stability of collagen's triple-helical structure in mammals requires prolyl-4-hydroxylase (P4H) activity to form hydroxyproline residues within the collagen chains. Although plants are capable of synthesizing hydroxyproline-containing proteins, plant-derived prolyl hydroxylase exhibits relatively loose substrate sequence specificity in comparison to mammalian P4H.[6,7] Coexpression of collagen and mammalian-derived prolyl-hydroxylase in insect cells,[8] yeast[9,10] and plants[11] enabled the formation of stable, hydroxylated collagen.

Further posttranslational modifications of collagens involve the lysyl hydroxylase, galactosyltransferase and glucosyltransferase enzymes which sequentially modify lysyl residues to hydroxylysyl, galactosylhydroxylysyl and glucosylgalactosyl hydroxylysyl, respectively. These lysyl carbohydrate structures are unique to collagens[12] and have been implicated in the control of fibril diameter.[13] The human enzyme Lysyl hydroxylase 3 (LH3) can consecutively catalyze all three modification steps required for hydroxylysine-linked carbohydrate formation.[12] In

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contrast, amino acid analysis of tobacco-expressed human collagen[11] demonstrated hydroxylysine content to be less than 2% of that found in bovine collagen, suggesting that endogenic plant lysyl hydroxylase is unable to sufficiently hydroxylate collagen lysines.

To date, collagen products used in pharmaceutical or biotechnological applications are extracted from animal or cadaver sources. The use of such materials involves several risks including allergenicity and potential contamination with pathogens. The scope of this work was to achieve high output of hydroxylated, heterotrimeric, recombinant human procollagen type I (rhPCOL1) in plants by coexpression of human procollagen alpha 1 and alpha 2 chains together with human posttranslational modifying enzymes P4H alpha, P4H beta, and LH3. Procollagen and P4H expression were targeted to subcellular compartments lacking similar endogenous plant enzymes or probable P4H inhibitors. Our results display efficient expression and purification of fibril-forming, thermally stable, heterotrimeric recombinant human collagen (rhCOL1). In addition, rhCOL1 served as a biologically active substrate for the attachment and expansion of endothelial progenitor-like cells isolated from adult human peripheral blood.

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Experimental Section

Constructs. All coding sequences in this work were optimized for expression in tobacco plants and chemically synthesized with desired flanking regions (SEQ ID Nos.: 1, 4, 7, 12, 14, 16, 18, 20, 22[14]). The genes coding for alpha 1 and alpha 2 procollagen chains (SEQ IDs 1, 4[14]) fused to either the vacuolar-targeting signal (SEQ ID No.: 24[14]) or to the apoplast-targeting signal (encoded by SEQ ID No.: 7[14]) or, without any targeting signal, were cloned into separate expression cassettes (pUC18) for each signal in question. The pUC18 plasmid included a *Chrysanthemum* rbcS1 promoter and 5′ UTR (SEQ ID No.: 10[14]) and a *Chrysanthemum* rbcS1 3′UTR and terminator (SEQ ID No.: 11[14]). The complete expression cassettes (promoter, coding region, and terminator) were cloned in the multiple cloning site (*Hin*dIII) of the pBINPLUS plant transformation vector.[15]

The genes encoding human-P4H beta and human-P4H alpha (SEQ ID Nos.: 12 and 14[14]) lacking the native C-terminal ER retention signal fused to either the vacuolar-targeting (SEQ ID No.: 24[14]) or the apoplast-targeting signal (encoded by SEQ ID No.: 7[14]) or without any targeting signal were cloned into expression cassettes composed of the CaMV 35S promoter and TMV omega sequence and *Agrobacterium nopaline* synthetase (NOS) terminator carried by the vector pJD330.[16] The complete expression cassettes were cloned in the multiple cloning site (XbaI) of the pBINPLUS vectors. The synthetic gene encoding LH3 (SEQ ID No.: 22[14]) with flanking strawberry vein banding virus (SVBV) promoter (NCBI accession AF331666, REGION: 623.950, version AF331666.1 GI:13345788) and terminated by *Agrobacterium octopin* synthase (OCS) terminator (NCBI accession Z37515, REGION: 1344.1538, version Z37515.1 GI:886843) lacking target signals or, alternatively, fused to either the vacuolar-targeting or the apoplast-targeting signal was cloned in the multiple cloning site (*Eco*RI) of the pBINPLUS vector carrying the expression cassette of P4H beta.

Signal Peptides. (i) Vacuole signal sequence of barley gene for Thiol protease aleurain precursor (NCBI accession P05167 GI:113603) MAHARVLLLALAVLATAAVAVASSSSFADSNPIRPVTDRAASTLA (SEQ ID No. 24[14]).

(ii) Apoplast signal of *Arabidopsis thaliana* endo-1,4-β-glucanase (*Cel*-1, NCBI accession CAA67156.1 GI:2440033) RKSLIFPVILLAVLLFSPPIYSAGHDYRDALRKSSMA (SEQ ID No. 9[14] and encoded by SEQ ID No. 7[14]).

Plant Transformations. Procollagen alpha 1 and alpha 2 and P4H/LH3-transformed EHA 105 *Agrobacterium* was grown on YEB medium O.N with shaking at 28 °C. Following centrifugation, the culture was resuspended in MS medium (pH 5.8) to a final turbidity of 0.5 ($O.D._{600}$). The solution was poured on sterile miracloth (CalBiochem cat. No. 475855) immersed in water. Intact green leaves from 4 week old sterile tobacco plants were cut 3 to 4 times in a feather pattern, placed on the miracloth and then transferred to a sterile MS medium Petri dish containing indoleacetic acid (IAA) and kinetin. Following a 48 h incubation in the dark at 28 °C, leaves were transferred to new MS-containing Petri dish supplemented with IAA, kinetin, carbenicillin, and kanamycin for 3 week incubation at 28 °C in light. Fresh supplemented medium was then provided for an additional three weeks. The developed shoots were transferred to sterile MS medium-containing Petri dishes only supplemented with antibiotics. Following root growth, plants were transferred to nylon-covered pots to allow for maximum humidity and allowed to grow for three days.

Extraction and Purification of Collagen from Transgenic Plants. Tobacco leaves (450 g) were blended in chilled extraction buffer (900 mL of 100 mM Tris-HCl, pH 7.5 containing 4.5 mM potassium-metabisulfite and 7.5 mM EDTA), supplemented with 7.5 g polyvinylpyrrolidone (PVPP) and 3 g activated carbon. Blending was performed in five intervals of 1 min each, while temperatures were kept below 15 °C. Crude extracts were filtered through a gauze pad and centrifuged (30 min, 26000 g, 5 °C). The supernatant was collected, and $CaCl_2$ was added to a final concentration of 10 mM together with 1 g/L activated carbon. Propeptide cleavage was initiated by the addition of 5 mg/L ficin (Sigma #F4125; 15 °C, 3 h with stirring). Nonsoluble contaminants were then removed by centrifugation (30 min, 22000 g, 15 °C). rhCOL1 of the recovered supernatants was precipitated by gradually adding crystalline NaCl to a final concentration of 3.13 M (25 min, R.T with constant stirring). The solution was incubated in a cold room for 8 h O.N. without stirring. Collection of the rhCOL1-containing pellet was performed following centrifugation (26000 g, 2 h, 5 °C).

The pellets were then resuspended in a 200 mL solution of 250 mM acetic acid + 2 M NaCl for 5 min, using a magnetic stirrer, and then centrifuged (26000 g, 40 min, 5 °C). Supernatants were discarded and the pellet was resuspended in 200 mL of 0.5 M acetic acid (1 h, R.T.). Elimination of insoluble matter was performed by centrifugation (16000 g, 30 min, 15 °C). The supernatants were passed through 12 layers of gauze pad. rhCOL1 was then precipitated by slowly adding NaCl to a final concentration of 3 M with constant stirring for 20 min at R.T. The solution was incubated (8 h, O.N., 4 °C) and rhCOl1 was collected following centrifugation (26000 g, 2 h, 5 °C). All supernatant traces were removed. Pellet redissolving and rhCOL1 precipitation steps were repeated as above in acetic acid and NaCl solutions, respectively. Following the O.N. incubation and collection of rhCOL1-containing pellets, samples were redissolved in 40 mL of 10 mM HCl by vigorous pipettation and vortexing for 5 min at R.T. The solution was transferred to a dialysis bag (MWCO 14000 Da) and dialyzed against 4 L of 10 mM HCl (4 h, 4 °C). An additional dialysis was performed O.N. rhCOL1 was sterilized by filtering through a 0.2 μ filter using a 30 mL syringe. rhCOL1 was then concentrated using a Vivaspin PES 20 mL filtration tube (Vivascience, #VS2041, MWCO 100000). Centrifugation was performed until the volume was reduced to 0.75 mL (45 min, 5000 g, 5 °C).

Western Blot Analysis. Total soluble proteins were extracted from tobacco plant transformants by grinding 500 mg of leaves in 0.5 mL 50 mM Tris-HCl (pH 7.5) enriched with a protease inhibitor cocktail (Roche Diagnostics GmbH, product #1836145). The crude extract was boiled for seven minutes in 250 μL of 4X Sample application buffer (SAB) and centrifuged (8 min, 13000 rpm, R.T.). Supernatant samples (20 μL) were separated on a 10% polyacrylamide gel and proteins of interest were immunodetected using standard Western blot procedures. Detection of human P4H alpha and beta subunits and rhCOL1 type I alpha 1 and alpha 2 chains was effected using antihuman P4H alpha (ICN Biomedicals Inc., #63−163), antihuman P4H beta (Chemicon Inc., #MAB2701), and anticollagen I antibodies (Chemicon Inc., #AB745), respectively. Size markers were purchased from Fermentas Inc. (#SM0671).

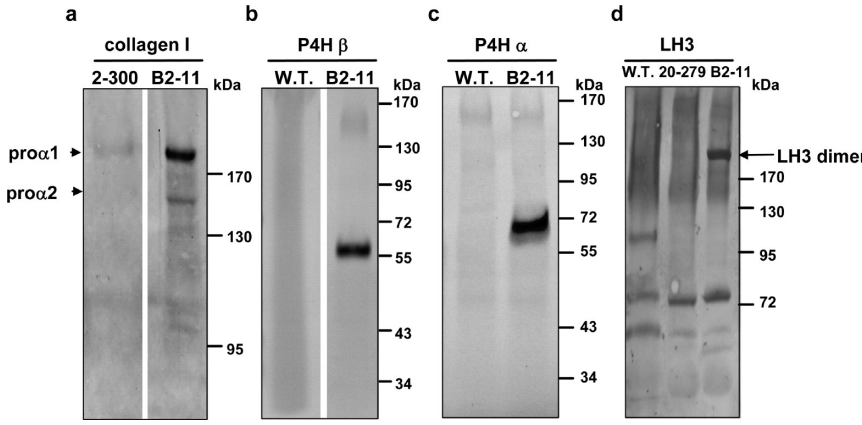

Figure 1. Expression of human rhPCOL1 alpha 1 and alpha 2 chains, P4H alpha, P4H beta, and LH3 in transgenic tobacco plants. Following transformation of parent plant lines (2−300 and 20−279) and their later crossbreeding to yield progenitor plant B2−11, total soluble protein extracts were prepared to verify expression of proteins of interest. Protein samples were separated on an 8% SDS-PAGE transferred to nitrocellulose membranes and immunoblotted for collagen I (a). Protein samples of B2-11 and 20-279 plant lines were prepared and separated on a 10% SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted for human P4H beta (b), human P4H alpha (c), and LH3 (d), with the respective antibodies as described in the methods section. Negative control samples were prepared from wild type nontransformed tobacco plant extracts (W.T.).

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Anti-LH3 Antibodies. Rabbit-anti-LH3 were raised by Genescript Inc., using standard methods,[17] against the following KLH (Keyhole Limpet Hemocyanin, SIGMA #H7017)-linked synthetic peptides representing LH3 epitopes: YRPDEQPSLRPHHDC and NQDRRTLPGGQPPPC.

Collagen Triple Helix Assembly and Thermal Stability. rhCOL1 triple helix stability was tested by a thermal denaturation technique followed by pepsin digestion according to the method developed by Bruckner and Prockop.[18] Total soluble proteins were extracted by grinding 500 mg B2−11 leaves in a chilled 0.5 mL solution of 100 mM Tris-HCl pH 7.5 and 300 mM NaCl and centrifuged (6 min, 10000 rpm, 4 °C). Supernatants (50 μL) were subjected to heat treatment for 30 min in the range of 23−41 °C. Samples were titrated to pH 2.5 with 250 mM HCl and incubated overnight at room temperature with 0.7 mg/mL pepsin. The digestion was terminated by adding 5 μL of unbuffered 1 M Tris. Each sample was mixed with 23 μL of 4× SAB, boiled for 7 min, and centrifuged (7 min, 13000 rpm). Supernatant samples (10 μL) were separated on a 10% polyacrylamide gel and collagen chains were immunodetected using standard Western blot procedures. Samples (2 μg) of human type I collagen (CalBiochem Inc.), extracted from human skin by pepsin digestion, were spiked into total soluble proteins from wild type tobacco extracts and served as positive controls.

DSC Analysis. rhCOL1 and commercial bovine collagen were analyzed (0.5 mg/mL in 100 mM sodium phosphate buffer and 0.5 M glycerol, pH 7.4) in a Microcal VP-DSC-ER calorimeter (Microcal Inc., Northampton, MA) at a scan rate of 30 °C/h in the range of 20−50 °C. The data was analyzed using the 5.0 software supplied by the DSC manufacturer.

Amino Acid Analysis of Purified rhCOL1. A total of 10−15 mg of rhCOL1 in 10 mM HCl was analyzed by Ansynth laboratories (Holland).

Monosaccharide Composition Analysis of Purified rhCOL1. Monosaccharide composition analysis of purified rhCOL1 was performed by glycosidic bond cleavage, followed by derivatization and GC-MS analysis (Mscan, U.K.).

C-Propeptide ELISA. Plant extract samples were placed over microtiter plates precoated with mouse monoclonal antiprocollagen type I C-propeptide (PIP) (Procollagen type I C- propeptide Elisa Kit: Takara, MK 101). Following incubation and extensive washing, plates were incubated with mouse monoclonal anti-PIP-peroxidase. Sample concentrations were determined by comparing their specific absorbance with the commercial kit standards.

Collagenase Assay. rhCOL1 (50 μL of 1 mg/mL) was suspended in 5 μL buffer (200 mM Na_2HPO_4, pH 11.2) and 5 mM $CaCl_2$ and then incubated with bacterial collagenase (1 μg, Sigma #C0773; 37 °C, O.N.). Proteins were separated on an SDS-PAGE following denaturation in 4× SAB.

Circular Dichroism (CD). CD measurements of 40 μg/mL rhCOL1 or 40 μg/mL human skin type I collagen (CalBiochem, U.S.A.) prepared in 10 mM HCl were performed using a JASCO Model J-810 spectropolarimeter (Jasco, U.K.) in a quartz cuvette (104-QS, Hellma, Germany) with a 10 mm path length. The cuvette was filled with 1 mL of sample for each measurement. CD spectra were obtained at room temperature by continuous wavelength scans from 200 to 260 nm at a scan speed of 50 nm per min. Averages of three scans per sample were calculated.

Preparation of Samples for SEM. Fibril formation was induced by mixing rhCOL1 (50 μL of 1 mg/mL) with 5 μL of fibrillogenesis buffer (200 mM Na_2HPO_4, pH 11.2) and incubating for 1 h, 37 °C. Fibrils were collected by centrifugation (5 min, 13000 rpm). Samples of collagen fibrils were then immersed in 0.1 M phosphate buffer (pH 7.2) and 2.5% glutaraldehyde (4 °C, O.N.). The samples were then rinsed five times in phosphate buffer and gradually dehydrated by adding increasing concentrations of ethanol (25−100%), then rinsed for 20 min, and finally dried in a Critical Point Dryer (Bio-Rad C.P.D 750). Samples were gold coated (coating thickness 25 nm) by E5150 sputter coater (Polaron Equipment, Ltd. England) and scanned by JSM-5410 microscope (JEOL Ltd. Japan).

Cell Isolation and Culture. Peripheral blood mononuclear cells (PBMNCs) were isolated from whole blood by density gradient centrifugation with Ficoll (Ficoll PaqueTM PLUS, Amersham Biosciences, Sweden). Isolated PBMNCs were seeded on plates precoated with 200 μg/mL rhCOL1 or 200 μg/mL native human collagen, diluted in human serum-containing medium, supplemented with human recombinant vascular endothelial growth factor (rVEGF, RELIATech, Germany). Unbound cells were removed by washing. Evaluation of the number of attached cells was performed using a Vi-Cell XR automatic cell counter (Beckman Coulter, U.S.A.). Statistical analysis: values are reported as mean ± SD. *t* test analysis was used to identify significant differences in the yield of attached cells by setting the significance level at $p = 0.05$.

Results and Discussion

Expression of Human rhPCOL1 alpha 1 and alpha 2 chains, P4H alpha, P4H beta, and LH3 in Transgenic Tobacco Plants. A transgenic plant line expressing rhPCOL1 was established by crossbreeding the alpha 1 and alpha 2 procollagen-expressing plant 2−300 (Figure 1a) with plant

20−279 expressing the three human post-translational modification enzymes, P4H alpha and beta and LH3 (Figure 1d). PCR and Western blot analyses were performed to verify genome insertion and protein expression.

To determine the appropriate sublocalization for collagen expression and modification, the above setup was performed in three subsetups, where each subsetup directed collagen and enzyme expression to specific subcellular compartments. In each subsetup, all five genes were fused to an identical sorting signal, directing them to the vacuole,[19] apoplast,[20] or cytosol. Apoplast-targeted procollagen was both expressed at relatively low levels and proved to be readily degradable (data not shown). Similarly, cytoplasm-directed procollagen expression failed to yield detectable protein accumulation (data not shown). Vacuole-targeted procollagen expression was the only targeting strategy to yield viable protein products, as determined by Western blot analysis (data not shown). Similar results were described by Wei et al.,[21] when targeting *A. niger* β-glycosidase expression to varying subcellular compartments in transgenic tobacco. Plant B2−11, a crossbreed product containing all five vacuole-targeted genes and displaying high collagen expression, was selected and propagated for further expression, isolation and analysis of rhPCOL1 (Figure 1a−d). All further experiments were based on rhPCOL1 purified from plant B2−11. Of the plants expressing vacuole-targeted rhPCOL1, high levels of protein expression were only detected in plants coexpressing procollagen with both P4H alpha and P4H beta (plant B2−11, Figure 1a), supporting findings previously reported in insect, yeast and tobacco models.[8−11]

Recombinant LH3 proteins expressed in the B2−11 transgenic plant (Figure 1d) migrated above 170 kDa. While the calculated size of the LH3 monomer is 86 kDa, the protein has been reported to be active in vivo as a homodimer,[22] where the ~170 kDa band seemingly represents an LH3 dimer. These bands were not detected in the LH3-expressing 20−279 plant line, which was not transfected with procollagen-coding vectors. Thus, the presence of the collagen triple helix in plant B2−11 may be necessary for the formation of stable LH3 homodimers. Monosaccharide composition analysis of rhCOL1 purified from plant B2−11 demonstrated a yield of 0.276 μg galactose and 0.1924 μg glucose per mg collagen. This finding suggests glucosylgalactosyl activity toward hydroxylysines effected by the introduced human LH3 gene.

ELISA-based detection of C-propeptide led to estimations of rhPCOL1 expression to constitute 2% of the total soluble protein (TSP) in plant B2−11 or 200 mg rhPCOL1 per kg fresh leaves. These calculated vacuole-targeted collagen yields are substantial when compared to 0.1−1% TSP yields of nuclear-targeted protein expression in plants.[23,24] Earlier studies have described expression of N-propeptide-free homotrimeric collagen in tobacco plants[6,11] in a model which involved C-propeptide cleavage by plant-based proteolytic activity in mature plants. Final expression levels were approximated at 20 mg N-propeptide-free procollagen per kilogram leaves. In contrast, the approach presented here demonstrates accumulation of vacuole-targeted, full-length, heterotrimeric procollagen with intact C- and N-propeptides at 10-fold higher levels, thereby introducing a means of mass production of both procollagen and collagen at practical levels.

Triple Helix Assembly and Thermal Stability of rhPCOL1. The thermal stability of the B2−11-derived rhPCOL1 in comparison to that of human skin-derived collagen was tested by thermal denaturation followed by pepsin-induced cleavage (Figure 2a). Such digestion converts rhPCOL1 to rhCOL1 by cleavage of the propeptides and removal of all unfolded regions, thereby

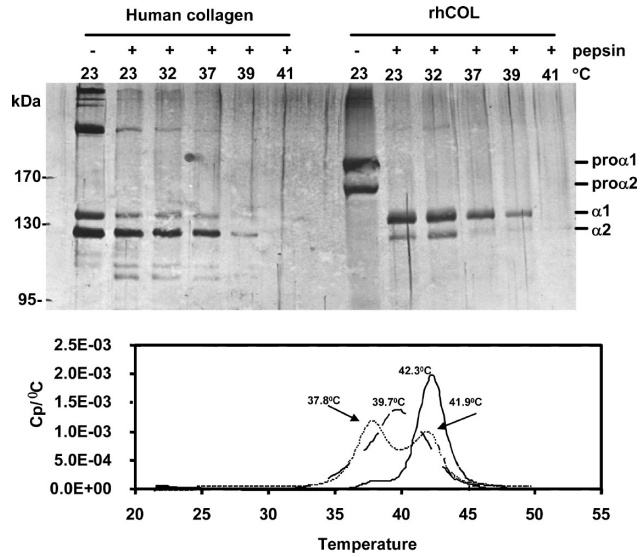

Figure 2. Determination of rhPCOL stability (a) B2−11-derived rhPCOL1 samples were heated to the indicated temperatures for 30 min followed by digestion with pepsin (0.7 mg/mL). The samples (10 μL/lane) were separated on a 8% SDS-PAGE in parallel to a heat-treated and pepsinized human skin collagen sample (2 μg/lane). Following transfer to nitrocellulose membranes, collagen was detected with anticollagen I. (b) DSC analysis of rhCOL1 (dashed line) was performed in parallel to pure bovine collagen purchased from Purecole (solid line) and Apcoll (dotted line). The three protein samples were scanned (30 °C/h) at a concentration of 0.5 mg/mL in a Microcal VP-DSC-ER calorimeter.

reflecting the resistance of helical rhCOL1 to protease activity. The gradual decrease in the quantity of the detected collagen is due to the fact that the extent of hydroxylation can vary from one collagen molecule to the other, resulting in a population of triple helices with different melting point temperatures. B2−11-derived collagen triple helices remained stable and resistant to denaturation and proteolysis up to 39 °C, mimicking the thermal stability demonstrated by control human skin collagen samples. In contrast, nonpost-translationally modified collagen helices, derived from plants 2−300 and 2−372, were thoroughly digested and denatured at temperatures as low as 23 °C (data not shown) displaying inferior collagen stability when relying on plant P4H for collagen hydroxylation. Furthermore, differential scanning calorimetry (DSC) analysis of the purified rhCOL1 (Figure 2b) demonstrated a melting point of 39 °C, which stands in accordance with the DSC value measured for pepsin-digested bovine collagen. These results show that coexpression of human P4H enzymes with collagen proved to be essential for rhPCOL1 and rhCOL1 conformation and stability.

Determination of Purity of B2−11-Derived rhCOL1 Samples. Collagenase assays were performed on B2−11-purified rhCOL1 samples to validate the identity of the purified protein by sample sensitivity to collagenase activity. In addition, the assay can demonstrate the presence of collagenase-insensitive contaminating proteins. High sample purity was determined after all visible bands of B2−11-purified rhCOL1 were digested following incubation with collagenase (Figure 3, lane 3 vs 7).

Protein Sequencing and Amino Acid Composition Analysis of B2−11-Purified rhCOL1. To further verify the identity of the expressed rhCOL1, rhPCOL1 samples were digested with fig-derived ficin protease and further purified to yield rhCOL1, which mimicked the migration of pure human placental type I collagen samples (Figure 4). Following electrophoretic separation of the purified rhCOL1, protein sequencing[25,26] was performed on the bands, which were thought

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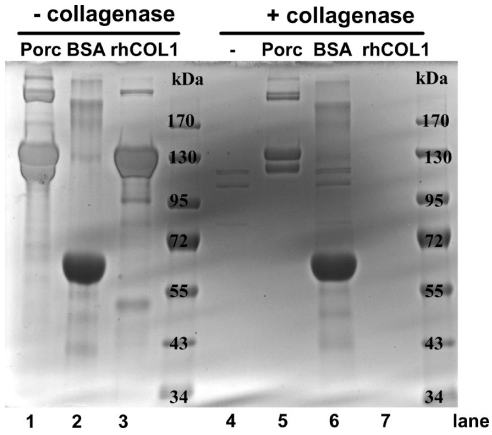

Figure 3. Determination of B2−11-derived rhCOL1 sample purity rhCOL1 (50 μg), porcine-derived collagen (Porc), and BSA were incubated in the presence or absence of 1 μg collagenase (37 °C, O.N). Samples were separated on a 10% SDS-PAGE and bands were detected with Coomassie blue staining solution.

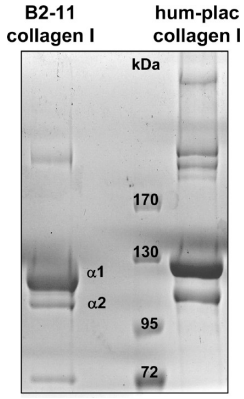

Figure 4. Verification of identity of protein bands of purified rhCOL1 samples Propeptides of B2−11-derived rhPCOL1 were cleaved by addition of 5 mg/L ficin to samples which were then further purified and separated on an 8% SDS-PAGE and stained with Coomassie blue staining solution. Pure human placental type I collagen was loaded as a positive control sample.

to correspond to alpha 1 and alpha 2 type I collagens (LC-MS/MS, data analyzed by Sequest 3.2 software). The bands indicated in Figure 4 were identified as alpha 1 type I collagen preproprotein (*Homo sapiens*; $p = 1.00 \times 10^{-30}$) and alpha 2 type I collagen (*Homo sapiens*; $p = 1.59 \times 10^{-12}$). All identified peptides (70% sequence coverage) displayed 100% identity to human collagen protein sequences.

Amino acid analysis of B2−11-derived rhCOL1 showed significant identity to the human-extracted type I collagen heterotrimer[27] (Table 1). Additionally, the hydroxylysine content was 37-fold higher than the levels detected in LH3-free tobacco plants,[11] establishing heterologous LH3 activity. Measured percentages of hydroxyproline content (7.55%) were similar to those reported for recombinant plant-derived collagen (8.41%)[11] and hydroxylysine content (0.74%) to those of human collagen (1%).[27]

Structural Analysis of rhCOL1. Circular dichroism (CD) analysis of rhCOL1 (Figure 5) yielded comparable spectra to that of human skin type I collagen (CalBiochem, U.S.A.), confirming similar structural conformation between rhCOL1 and human skin collagen. To visualize the plant-derived rhCOL1 fibril lattice network, B2−11-purified rhCOL1 was allowed to assemble to fibrils (1 h, 37 °C) that were then collected and analyzed by scanning electron microscopy (SEM; Figure 6).

Table 1. Amino Acid Analysis of B2-11-Derived rhCOL1 vs Human-Derived Collagen Heterotrimers[a]

amino acid	plant-derived rhCOL (%)	human collagen (%)
Asp+Asn	4.78	4.3
hydroxyproline	7.55	10.3
threonine	1.86	1.7
serine	3.27	3.3
Glu+Gln	7.64	7.1
proline	15.03	12.0
glycine	32.32	33.5
alanine	11.36	11.1
valine	2.29	2.6
isoleucine	0.92	0.9
leucine	2.55	2.3
tyrosine	0.36	0.2
phenylalanine	1.35	1.2
hydroxylysine	0.74	1.0
lysine	2.85	2.3
histidine	0.31	0.6
arginine	4.81	5.0
cysteine	ND	ND
methionine	ND	0.6
tryptophan	ND	ND

[a] See reference 27 {Nokelainen, 2001}.

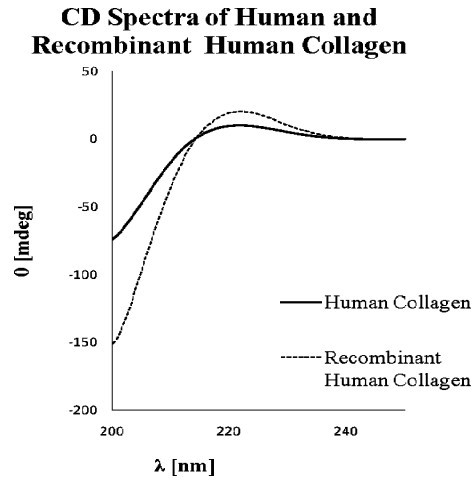

Figure 5. Analysis of structural conformation of rhCOL1. CD measurements of 40 μg/mL rhCOL1 (dashed line) and 40 μg/mL human skin type I collagen (solid line) prepared in 10 mM HCl were performed using a JASCO Model J-810 spectropolarimeter in a quartz cuvette with a 10 mm path length. The cuvette was filled with 1 mL of sample for each measurement. CD spectra were obtained at room temperature by continuous wavelength scans from 200 to 260 nm at a scan speed of 50 nm/min. Averages of three scans/sample were calculated and plotted as ellipticity (θ [mdeg]) vs wavelength (λ [nm]).

Long homogeneous fibrils and lattice structures characteristic to collagen were observed, indicating proper structure of the B2−11-derived rhCOL1.

Isolation and Growth of Peripheral Blood-Derived Human Endothelial Progenitor Cells (EPC) on B2−11-Derived rhCOL1. The attachment of endothelial cells to collagenous extracellular matrix proteins may be exploited for the isolation and enrichment of such cells from biological fluids.[28] To determine biofunctionality of rhCOL1, isolated peripheral blood mononuclear cells were seeded on plates precoated with B2−11-derived rhCOL1. EPC yields obtained using rhCOL1-coated plates were comparable to those obtained with tissue-derived human collagen (Figure 7b) and were several fold higher than those isolated from uncoated plates (Figure 7a). Moreover, rhCOL1 was as effective in isolation of cells from blood samples containing either very low or high endogenous levels of EPC (Figure 7a and b, respectively). The majority of cells isolated and grown on rhCOL1 appeared as

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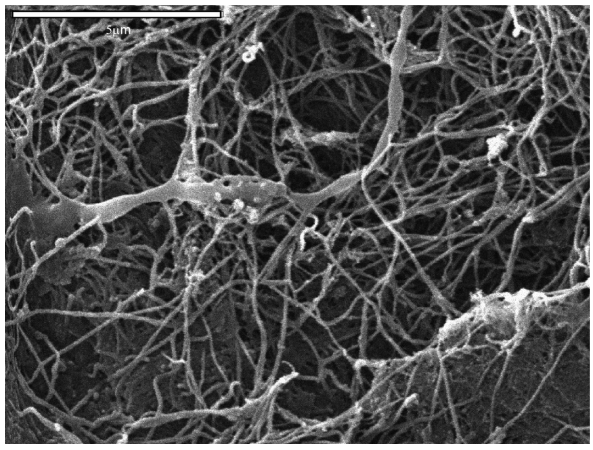

Figure 6. Scanning electron microscopy of B2−11-purified rhCOL1. B2−11-purified rhCOL1 (1 mg/mL) were allowed to assemble to fibrils by incubation (1 h, 37 °C) in fibrillogenesis buffer. Fibrils were collected and then immersed in 0.1 M phosphate buffer (pH 7.2) and 2.5% glutaraldehyde (4 °C, O.N.). Following extensive washings, samples were dehydrated by gradually adding increasing concentrations of ethanol (25−100%). Following rinsing and thorough drying, samples were mounted on metal stubs and gold coated. Magnification ×10000, scale bar ~5 μm.

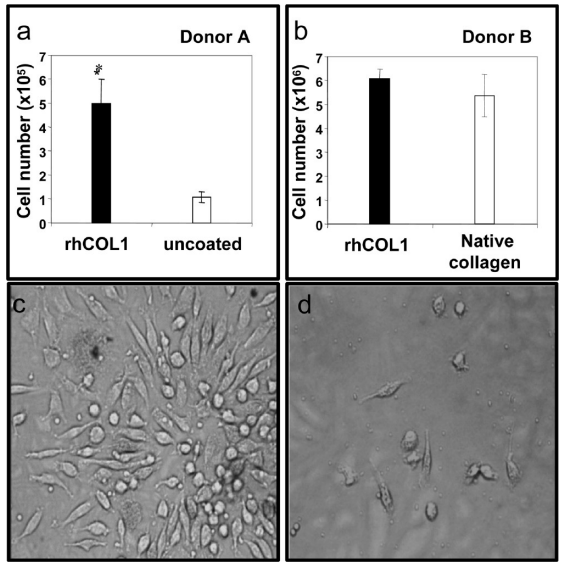

Figure 7. Bioactivity of rhCOL1. Freshly isolated PBMNCs were seeded on uncoated plates or plates coated with either 200 μg/mL rhCOL1 or native human collagen. Cells were counted after 7 days in culture. Results from two donors, with low (a) and high (b) numbers of EPC-like cells, are presented as mean values ± SD, $^*p < 0.05$, when compared to uncoated plates. Photomicrographs (×400) of typical elongated spindle-shaped cells, isolated on rhCOL1-coated plates (c) or uncoated plates (d) are shown.

typical spindle-shaped cells supported by strong interactions with the rhCOL1 matrix (Figure 7c), while cells grown on uncoated plastic plates were mostly round (Figure 7d). Similar observations were noted with cells derived from five unrelated donors (data not shown). These results display that the biological activity of rhCOL1 proved similar to human tissue-derived collagen through its capacity to support attachment and proliferation of adult peripheral blood-derived endothelial progenitor-like cells.

The relative ease associated with transformation and optimization of recombinant protein expression in tobacco plants, together with the ability to cross between plant lines to achieve concerted expression of multiple genes was demonstrated in this study. The high levels of rhPCOL1 expression and viable rhCOL1 isolated in this work paves the way to broadened applications of rhCOL1 in regenerative medicine.

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