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Hydroxylated human homotrimeric collagen I in *Agrobacterium tumefaciens*-mediated transient expression and in transgenic tobacco plant

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Abstract Potential contamination of animal-derived collagen with pathogens has led to the demand for safe recombinant sources of this complex molecule. In continuation of our previous work [Ruggiero et al. (2000) FEBS Lett. 469, 132–136], here we show that it is possible to produce recombinant hydroxylated homotrimeric collagen in tobacco plants that are co-transformed with a human type I collagen and a chimeric proline-4-hydroxylase (P4H). This is to our knowledge the first time that transient expression in tobacco was used to improve the quality of a recombinant protein produced in plants through co-expression with an animal cell-derived modifying enzyme. We demonstrated the functionality of the new chimeric P4H and thus improved the thermal stability of recombinant collagen I from plants to 37°C. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Recombinant collagen; Prolyl-4-hydroxylase; Transgenic plant; Transient expression

1. Introduction

Collagen I is widely exploited for medical, cosmetic and therapeutic uses. However, this material is mainly extracted from animal tissues, which entail potential viral or other infectious agent contamination. Development of alternative sources of collagen is thus attractive to the pharmaceutical industry. The use of transgenic plants as an alternative expression system for heterologous proteins represents a cost-effective large-scale production system [2,3].

We have previously shown that tobacco plants are capable of accumulating recombinant collagen (rColl I) as a fully processed triple helix [1]. However, the thermal stability of this triple helix was reduced compared to bovine collagen [1]. Amino acid analysis of recombinant plant collagen demonstrated a lack of hydroxyproline residues that are necessary to stabilize the structure of the trimeric molecule. Plants possess endogenous proline-4-hydroxylases (P4Hs), but these cannot ensure the correct prolyl hydroxylation in the repeating Xaa–Pro–Gly collagen sequence, where Xaa is an amino acid other than glycine [1,4]. The production of hydroxylated collagen in other recombinant systems such as insect cells and more recently yeasts has required the co-expression of P4H subunits [5–8]. Expression of human and chimeric P4H has been demonstrated in insect cells [9]. While mammalian P4H is a tetramer of two α and two β subunits, an α *Caenorhabditis elegans*/β human chimeric P4H was found to be an αβ dimer but appeared to have the same specific activity as the human P4H. The catalytic site is on the α subunit. Compared to the human enzyme, the chimeric enzyme was not inhibited by poly(L-proline). This observation could be an advantage for the expression of a chimeric enzyme in plant cells, since extensine molecules that are substrates of plant prolyl-4-hydroxylase are rich in Ser–$(Pro)_4$–Ser–Pro–Ser–$(Pro)_4$ sequences [10] and thus could inhibit P4H of mammalian origin. In addition, the nematode prolyl-4-hydroxylase is adapted to work at a lower temperature as compared to mammalian enzymes. We therefore opted to test the functionality of a similar enzyme, an α *C. elegans*/β mouse P4H in plant cells.

Here, for the first time, a rapid transient assay employing *Agrobacterium tumefaciens* infiltration was used to show in vivo biocatalysis in plant cells, by co-expression of the two recombinant proteins and purification of the resultant hydroxylated collagen (trOHColl I). Stable transformed tobacco plants were also obtained and the amino acid composition of purified rOHColl I was compared with the product from transient assays. These experiments demonstrate the capacity to introduce catalytic activities of animal origin into plants to improve the quality of the resultant recombinant proteins.

2. Materials and methods

2.1. Plasmid construction

2.1.1. Human collagen ΔNproα1(I) cDNA. Plasmid 1297 was constructed by inserting a *Hin*dIII filled-in DNA fragment isolated by digesting plasmid pBIOC-PRSα1(I) encoding a recombinant form of human procollagen α1(I) lacking its N-propeptide (ΔNpro) [1], between the promoter L3 derived from L2 and the nopaline synthase (TNOS) terminator [11]. The collagen expression cassette (promoter–gene–terminator) was isolated by digestion with *Mlu*I–*Hpa*I and cloned into the plant binary vector pMRT1195 to give the expression plasmid pMRT1279 [12]. A schematic representation of procollagen α1(I) and the ΔNproα1(I) collagen construct lacking the N-propeptide are illustrated in Fig. 1A.

2.1.2. Prolyl-4-hydroxylase cloning. C. elegans P4H α subunit. A

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750 bp DNA fragment corresponding to the 5′ region of the cDNA encoding the α subunit of P4H was amplified by PCR from a *C. elegans* λ cDNA library (Stratagene) using the oligonucleotides O676 (5′-AATAGAATTCCCCGGGAACAATGCGCCTGGCACTCCTT-3′) and O669 (5′-CTTCTTGCTCGAGAAGGTCCTCGTA-3′). Using oligonucleotides O668 (5′-TACGAGGACCTTCTCGAGCAAGAAG-3′) and O656 (5′-ATTTGATATCGTCGACTTAGAGGGTCTCCCAGAC-3′), the 930 bp of the 3′ region were amplified by PCR. The two PCR fragments were fused and cloned into pBluescript SK+ (Stratagene) using the restriction sites *Eco*RI, *Xho*I and *Sal*I that are underlined in the oligonucleotide sequences to re-create the full-length cDNA (1677 bp) encoding the P4H α subunit from *C. elegans* (plasmid pMRT1300). Plasmid pMRT1295 was constructed by introducing the full-length αP4H DNA, as a *Sma*I/*Apa*I filled-in fragment between the promoter pr1287 derived from 1139 and the terminator TNOS [13]. From plasmid pMRT1295, a *Mlu*I/*Pac*I DNA fragment corresponding to the expression cassette was cloned into *Mlu*I/*Pac*I-digested plasmid pMRT1279, in the opposite sense as the collagen expression cassette to give the plant expression plasmid pMRT1286. This construct was introduced into *A. tumefaciens* LBA4404 to give strain A1286. The T-DNA transferred from *A. tumefaciens* is shown in Fig. 1B.

Mouse P4H β subunit. A 1200 bp DNA fragment corresponding to the 5′ region of the cDNA encoding the β subunit of mouse P4H, was amplified by PCR from a Quick clone mouse kidney cDNA library (Clontech) using oligonucleotides O651 (5′-AATACCCGGGAACAATGCTGAGCCGTGCTTTGCT-3′) and O691 (5′-GGGGCATAGAATTCAACAAACACG-3′). A 350 bp DNA fragment corresponding to the 3′ region of the P4H β subunit cDNA was amplified by PCR using oligonucleotides O684 (5′-TGTTGAATTCTATGCCCCTTGGTGTGGT-3′) and O683 (5′-ATTTGGTACCGAGCTCCTACAGTTCATCCTTCACAGCT-3′). The two PCR fragments were fused and cloned into pBluescript SK+ (Stratagene) using the restriction sites *Sma*I, *Eco*RI and *Kpn*I that are underlined in the oligonucleotide sequences to re-create the full-length cDNA (1550 bp) encoding the P4H β subunit from mouse (plasmid pMRT1302). The cDNA was placed under the control of the promoter 1287 by subcloning into plasmid pMRT1287 using the *Sma*I/*Kpn*I sites, to give plasmid pMRT1294. The resulting expression cassette was inserted into the plant expression plasmid pMRT1279, in the opposite sense from collagen using *Mlu*I/*Pac*I to create plasmid pMRT1284. This construct was introduced into *A. tumefaciens* to obtain strain A1284. The T-DNA is shown in Fig. 1B.

2.2. *A. tumefaciens-mediated transient assay*

Tobacco plants were agroinfiltrated according to the protocol of Kapila et al. [14] with one or with two *A. tumefaciens* strains simultaneously.

2.3. *Plant transformation*

Tobacco leaf discs were transformed with *A. tumefaciens* strains A1286 and A1284 simultaneously [15]. Transformed plants were selected with glufosinate at a concentration of 0.5 mg/ml for 4 weeks, 2 mg/ml for 3 weeks and finally 1 mg/ml for 3 weeks. Plants were kept in vitro until sufficiently mature to be transferred to the greenhouse. Leaf material was collected for protein analysis at 10 weeks after transfer to the greenhouse. The plants were grown to maturity and the seeds were collected.

2.4. *Transgenic tobacco selection*

Three-month-old glufosinate-resistant plantlets were screened by PCR, to detect plants possessing all three transgenes (collagen and both P4H subunits). DNA was extracted from in vitro plantlets using the CTAB method [16]. A multiplex PCR was performed with a thermocycler (Perkin Elmer), using the following primers: for collagen O820 (5′-GGCAGCACCAGTAGCACCATCATTT-3′); for the L3 promoter O819 (5′-GTATGCCGGTTCCCAAGCTTTATTTCC-3′); for the P4H α subunit O669 (5′-CTTCTTGCTCGAGAAGGTCCTCGTA-3′) and for the 1287 promoter O834 (5′-ATGACGCACACGCAGCCATGGTCCTGAA-3′); for the P4H β subunit O821 (5′-GCCAGCTGTATATTCCTTTGGGGAGGCT-3′) and O834. Three PCR fragments of 457 bp for the β P4H subunit, 691 bp for collagen and 884 bp for the α P4H subunit were amplified from plants, which had incorporated the three genes.

2.5. *Extraction and purification of recombinant collagen*

Tobacco leaves from agroinfiltrated plants or stable transgenic plants were collected and stored at −80°C. Before extraction, the leaves were frozen in liquid nitrogen and homogenized with IKA Universalmühle M20. Total protein was extracted, at a ratio of 3 ml/g in 500 mM acetic acid, 200 mM NaCl, 1 mM EDTA, pH 2.8, at 4°C, for 30 min and centrifuged (15 000×*g*) to eliminate insoluble materials, as described in [1]. Collagen was precipitated with 0.9 M NaCl overnight at 4°C. The precipitate was centrifuged and the pellet was dissolved in 1 ml of 100 mM acetic acid.

2.6. *Characterization of recombinant collagen*

The 0.9 M NaCl precipitate containing the recombinant collagens rColl I, trOHColl I and rOHColl I was purified by reverse phase high-performance liquid chromatography (HPLC) on Uptisphere 5μWOD C18 (50×2 mm) (Waters) using an aqueous acetonitrile gradient in the presence of 0.1% trifluoroacetic acid (TFA), with bovine Coll I homotrimer as a hydroxylated control [17].

The amino acid composition of all collagen samples was determined after hydrolysis under vacuum (6 N HCl, 115°C, 24 h) in the presence of 2-mercaptoethanol in a Pico Tag system (Waters) with a Beckman amino acid analyzer.

Purified rOHColl I was incubated for 6 min in a Tgradient thermocycler (Biometra) at either 30.5°C, 31.7°C, 33°C, 34.3°C, 35.6°C, 37°C, 38.3°C, 39.5°C, or 40.2°C, then treated for 30 min at 22°C with pepsin (0.1 mg/ml) [18]. As a control, collagen was incubated without pepsin. Proteolysis was stopped with Laemmli loading buffer and incubation for 5 min at 95°C, the samples were analyzed by SDS–PAGE in a 6% gel, and stained with Coomassie brilliant blue R250. The band corresponding to collagen at 120 kDa was quantified using a GS710 calibrated imaging densitometer (Bio-Rad).

Circular dichroism analysis of rColl I, rOHColl I and bovine Coll I homotrimer was performed as described in [1].

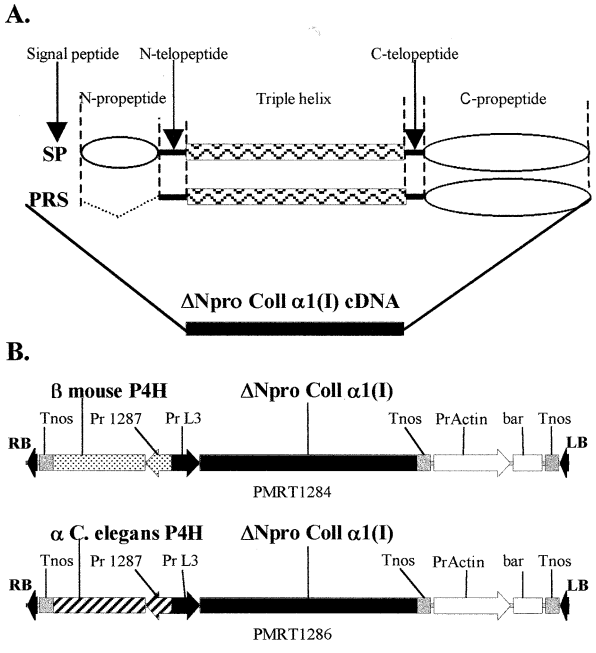

Fig. 1. A: Schematic presentation of human procollagen α1(I). The signal peptide (SP) is replaced by the plant signal peptide (PRS) and the N-propeptide is deleted to obtain the ΔNproColl α1(I) cDNA fragment [1] for plant transformation. B: T-DNA segments containing the collagen and P4H expression cassettes. The T-DNA from pMRT1284 encodes ΔNproColl α1(I) under the control of the L3 promoter (Pr L3) and β mouse P4H cDNA with its native signal peptide under the control of the 1287 promoter (Pr1287). The T-DNA from pMRT1286 encodes ΔNproColl α1(I) under the control of the L3 promoter (Pr L3) and α *C. elegans* cDNA with its native signal peptide under the control of the 1287 promoter (Pr1287). RB, right border; LB, left border of T-DNA. Bar under control of the actin promoter (Pr Actin) is the resistance gene. TNOS is the transcriptional terminator used in all constructs.

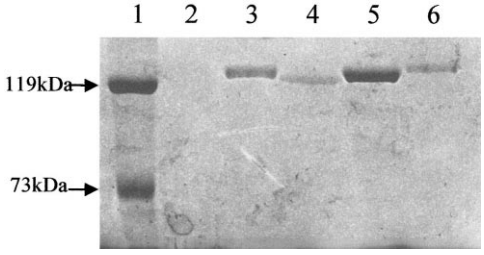

Fig. 2. Purified collagen on 6% SDS–PAGE, Coomassie blue staining: lane 1: MWM Prosieve® color protein markers, BioWhittaker Molecular Applications; lane 2: non-transgenic tobacco control; lane 3: rOHColl I; lane 4: rColl I; lane 5: trOHColl I; lane 6: bovine Coll I homotrimer.

3. Results

3.1. Transient co-expression of homotrimeric collagen type I and of an active chimeric P4H

A transient expression system was employed to study the co-expression of collagen and chimeric P4H in tobacco plants. This technique provides a fast (e.g. days as opposed to months for stable plant regeneration) method to assess the feasibility of the expression of foreign proteins in plants.

Recombinant collagen was extracted 4 days following vacuum infiltration of tobacco plant material with two *A. tumefaciens* strains: A1286 (containing T-DNA encoding ΔNproα1(I) collagen and the *C. elegans* P4H α subunit) and A1284 (ΔNproα1(I) collagen and mouse P4H β subunit) (Fig. 1). The purified trOHColl I was analyzed by 6% SDS–PAGE under reducing conditions. The collagen specific band of approximately 120 kDa migrates almost at the same level as bovine Coll I homotrimer control but slightly higher than rColl I non-hydroxylated collagen (Fig. 2, lanes 4–6). We used bovine homotrimer as a control for the recombinant homotrimers, since human homotrimer is difficult to obtain and we preferred not to compare the recombinant homotrimer with a human Coll I heterotrimer [1,17]. The specificity of the bands was confirmed by Western blotting using polyclonal antibodies against human collagen I (data not shown). These results

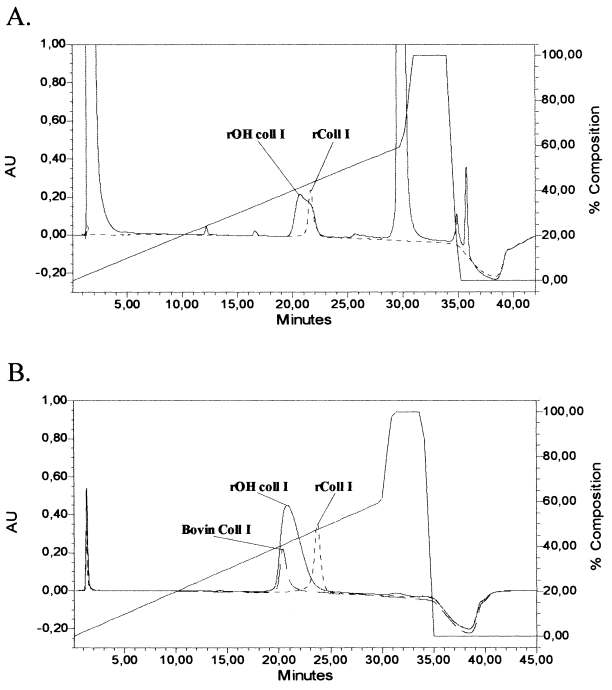

Fig. 3. HPLC elution profiles. A: Solid line: trOHColl I; dotted line: rColl I. B: Solid line: rOHColl I; dotted line: rColl I; dashed line: bovine Coll I homotrimer. % Composition: % in elution buffer B (80% acetonitrile, 0.8% TFA in water).

indicate that transiently transformed tobacco plants provide a good system to produce spontaneously processed collagen molecules without using pepsin treatment as already described for stable tobacco [1]. The collagen concentration in the purified preparation was about 40 μg/ml, resulting in a yield of approximately 50–100 μg of purified collagen per 100 g of agroinfiltrated leaf material.

Analysis by reverse phase HPLC showed that trOHColl I extracted from co-transfected leaves eluted in two fused peaks (Fig. 3A), of which only the second peak had the same retention time as non-hydroxylated rColl I control. The first

Table 1
Amino acid composition (indicated values are means of two independent experiments)

Amino acid	Transient expression trOHColl I residues (%)	Stable plant rOHColl I residues (%)	Stable plant rColl I residues (%)	Bovine Coll I homotrimer residues (%)
Hyp	6.84	8.41	0.68	10.24
Pro	15.8	14.58	21.71	11.43
Asp	4.35	4.11	4.2	4.33
Thr	1.58	1.65	1.64	2.03
Ser	3.56	3.48	3.5	2.62
Glu	7.68	7.76	7.89	9.21
Gly	33.4	32.89	32.75	33.2
Ala	11.24	11.95	12	10.73
Cys	0	0	0	0
Val	2.32	1.69	1.68	1.54
Met	0.83	0.64	0.61	0.89
Ile	0.57	0.55	0.58	0.82
Leu	2.2	1.82	1.9	2.43
Tyr	0	0.02	0.03	0
Phe	1.98	1.46	1.48	1.52
His	0.26	0.17	0.19	0.23
Hyl	0	0.04	0.02	1.88
Lys	3.6	3.5	3.61	1.64
Trp	nd	nd	nd	nd
Arg	3.77	5.28	5.52	5.35

elution peak could correspond to collagen with a higher hydroxyproline content. The presence of both hydroxylated and non-hydroxylated collagen in these tobacco leaves can be explained by the use of two *A. tumefaciens* strains for transformation. To produce hydroxylated collagen, T-DNA from both *A. tumefaciens* clones has to be transferred and to be expressed in the nucleus of tobacco cells. Cells that receive only one of the T-DNA constructs will not be able to synthesize a functional P4H dimer, thus producing only rColl I. Amino acid analysis of the collected trOHColl I revealed the presence of 6.85% hydroxyproline and 15.8% proline, as compared to 0.68% and 21.71%, respectively, for rColl I (Table 1). The samples contained 33% glycine residues, attesting to the purity of this collagen. This result showed for the first time hydroxylation of collagen by a chimeric *C. elegans*/mouse P4H in a plant expression system. Collagen from plants that were agroinfiltrated with A1286 alone (collagen plus α subunit) did not contain hydroxyproline residues (data not shown), which indicates that α subunit alone or in association with endogenous plant-derived β subunit (protein disulfide isomerase) is not functional in plant cells.

3.2. Stably transformed tobacco plants accumulate rOHColl I

To confirm the synthesis of hydroxylated collagen in transient plant expression and to produce more collagen, stable tobacco plants containing the collagen and chimeric P4H genes were produced. Plantlets resistant to the selecting agent were analyzed by multiplex PCR, to identify stably co-transformed plants. Of 71 plantlets, five had the three genes (collagen and both P4H subunits), 25 plantlets had only collagen and the β subunit, and there were no plants with collagen and α subunit. Reverse transcription-PCR was used to confirm the transcription of all three genes (data not shown) and rOHColl I was extracted from these plants only. The extraction yield varied from a maximum of 20 mg/kg of leaves to a minimum of 0.14 mg/kg. Purified rOHColl I was analyzed by SDS–PAGE (Fig. 2, lane 3), reverse phase HPLC and for hydroxyproline content. On SDS–PAGE the rOHColl I specific band of approximately 120 kDa migrates at the same level as trOHColl I and bovine Coll I homotrimer control (Fig. 2, lanes 5 and 6), but slightly higher than the rColl I non-hydroxylated collagen (Fig. 2, lane 3). HPLC reverse phase separation showed that rOHColl I in contrast to trOHColl I elutes in one peak, as bovine Coll I homotrimer, at an earlier time than rColl I (Fig. 3B). This indicates that stable plants produced only hydroxylated collagen as compared to the transient expression systems that contain both (cells expressing) hydroxylated and non-hydroxylated triple helical molecules (Fig. 3A). Amino acid analysis showed that rOHColl I extracted from selected plants contained 8.4% hydroxyproline (Table 1), while plants transformed with collagen alone (no P4H) integrated only 0.68% hydroxyproline into their collagen molecules. This confirms the data obtained in the transient assay, showing that the chimeric P4H is functional also in the stably transformed plants.

3.3. rOHColl I is thermostable at 37°C

To assess the thermal stability of the plant-derived rOHColl I, its sensitivity to pepsin was determined [18]. Using a temperature range between 30°C and 40°C we showed that purified rOHColl I was resistant to pepsin up to 36.8°C (50% degradation point as measured by scanning of collagen bands after PAGE) (Fig. 4A,B). To obtain more accurate data on the thermal stability of the different recombinant collagen molecules, circular dichroism spectra were performed. The spectra were typical for a triple helical conformation ([1] and data not shown). The thermal transition curve for rOHColl I measured by circular dichroism at 225 nm indicated a T_m value of 37.3°C at which 50% of the collagen molecules remain in a fully folded conformation as compared to 30°C for rColl I and 42°C for bovine homotrimer control (Fig. 4C). Micro-unfolding within the homotrimer control molecule could be responsible for the bi-phasic shape of the bovine collagen curve [19].

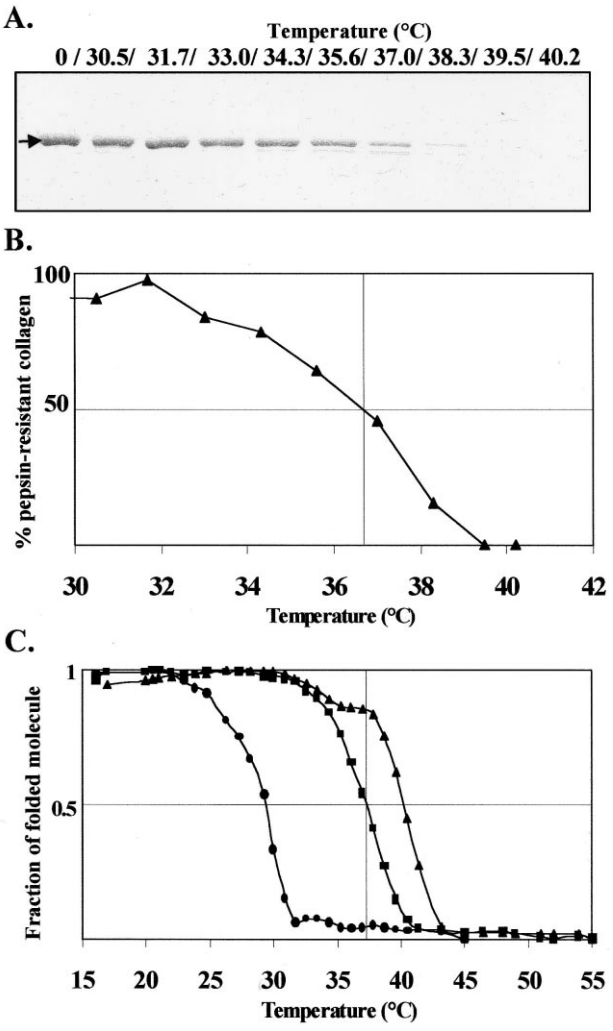

Fig. 4. Thermal stability of rOHColl I. A: SDS–PAGE of purified rOHColl I (→) incubated in a Tgradient thermocycler at temperatures between 0 and 40.2°C, then digested at 22°C with pepsin. B: The 120 kDa band corresponding to rOHColl I on 6% SDS–PAGE was quantified using a GS710 calibrated imaging densitometer, 100% corresponding to control value without pepsin digestion. C: Thermal transition curve of rColl I (●), rOHColl I (■), and bovine Coll I homotrimer (▲) as measured by circular dichroism at 225 nm.

4. Discussion

Plants have recently been used as an expression system for recombinant heterologous proteins [3,20,21]. However, in some cases the recombinant protein has been produced with-

out post-translational modifications that are important for its optimal function, thus hampering its final application. Our results demonstrate that it is possible to engineer successfully a new post-translational modification pathway in plants to overcome this problem.

Biocatalysis has been described in stable tobacco plants with the co-expression of human β1,4-galactosyltransferase resulting in galactosylation of plantibodies [22]. Hydroxylation of proline through the co-expression of P4H and collagen is a new example of biocatalysis in stable tobacco.

In transient expression, assembly of a full size mouse/human chimeric antibody has been demonstrated by simultaneous expression of the light and heavy chains after vacuum infiltration of tobacco leaves with two populations of recombinant agrobacteria, thus demonstrating that multimeric proteins could be transiently expressed in plants [23]. In this article, we employed transient expression with tobacco plantlets as a rapid test system to validate the co-expression strategy for the introduction of specific post-translational modifications. This shortens considerably the relatively long timelines involved in the development of stable plants when production of a small amount of complex proteins is required. Characterization of our recombinant protein from transient and stable expression showed for the first time the possibility to test biocatalysis in a transient assay prior to the generation of stable plants.

Previously we showed that rColl I can be expressed and produced in a triple helical form in transgenic tobacco [1]. In the present work, we expressed recombinant homotrimeric collagen I, together with animal-derived P4H, itself supposed to be a heterodimer, to specifically increase the hydroxyproline content, and consequently the thermal stability of the collagen triple helix.

Our data show that stable transgenic plants co-expressing the three genes were successfully obtained. We also transformed transgenic plants already expressing rColl I with *A. tumefaciens* containing the two cDNAs encoding the chimeric P4H (data not shown). In all cases hydroxylated rOHColl I was obtained. Hydroxyproline levels of 8.4% were identical to the 8.2% of hydroxyproline found in the recombinant type I collagen expressed in *Saccharomyces cerevisiae* [8]. This level is slightly below 9.6% of hydroxyproline residues found for a recombinant human type I homotrimer expressed in insect cells [7] or to the 10% hydroxyproline in *Pichia pastoris*-derived collagen I [6] or native human collagen. Compared to rColl I expressed in tobacco [1], the direct detection of hydroxyproline residues by amino acid analysis unequivocally demonstrated the functionality of the chimeric prolyl hydroxylase enzyme in tobacco plants. In addition, the reverse phase chromatography described here could be used in the future to detect rapidly differences in hydroxylation since the retention time of the hydroxylated collagen is different from that of non-hydroxylated collagen. The circular dichroism spectrum confirmed that the rOHColl I adopts the expected triple helix conformation and is thermostable with a T_m of approximately 37°C, which is in agreement with the hydroxyproline level.

The T_m of rOHColl I is 5°C below the T_m (42°C) determined for a type I collagen homotrimer expressed in insect cells [7] or a non-recombinant collagen. This difference of 1.2% in hydroxyproline content could explain the lower thermal stability of rOHColl I. Increasing the T_m value of plant collagen through co-expression of a chimeric P4H from 30°C to 37°C is an important advancement in relation to our previous work [1], concerning the quality and conformity of recombinant collagen from plants. Further improvements, such as increasing expression levels of rOHColl I in tobacco or expression of collagen in other plant species, are required for a biotechnological exploitation of this system.

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