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Original Article

Biolistic Transformation and Expression of Functional Chymosin from a Codon-Optimized Synthetic Bovine Gene in Tobacco Plants

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ABSTRACT

Chymosin is an important milk clotting enzyme, massively used in the dairy industry. Due to the limited amount of natural chymosin, the recombinant enzyme produced in different organisms is the main source of chymosin. Plants have several advantages for large scale cost-effective production of recombinant chymosin. Here, we used a synthetic codon-optimized version of bovine prochymosin gene for ideal expression in the tobacco host. In this study, we utilized biolistic co-transformation method to introduce the synthetic bovine prochymosin gene into the tobacco genome. Several transgenic plants were regenerated on a selective medium. Molecular analysis confirmed successful integration and expression of prochymosin gene in several transgenic candidates. Total soluble protein extracts from transgenic plants were successfully applied for milk coagulation experiments, demonstrating the production of functional prochymosin in transgenic lines. In conclusion, here, we report successful expression of functional chymosin in tobacco plants from a novel synthetic version of the bovine prochymosin gene. Our experimental data support that plants could serve as a reliable source for safe and cost-effective production of recombinant chymosin enzyme for industrial usage.

INTRODUCTION

Genetic engineering is a great tool that made it possible to transfer genes across species. Heterologous protein production is one of the central achievements of genetic engineering. To date, different heterologous proteins with various applications have been produced in different expression hosts by recombinant DNA technology [1-3]. Remarkably, more than 60% of industrial enzymes used in detergents, food, and starch processing industries, are made by recombinant DNA technology [4]. One of the important enzymes in the dairy industry is chymosin. This enzyme which is also known as rennin, is a protease produced by newborn ruminant animals to curdle the milk they ingest, resulting in a better absorption. The chymosin is found in a wide range of tetrapods, however, bovine chymosin has a high clotting activity, and therefore, is widely used for cheese production [5]. The natural sources of chymosin can not fulfill the requirements of rapidly growing cheese industries. As an altenative, recombinant chymosin is largely produced in different heterologous hosts [6-8]. Microbial systems are commonly used for the production of recombinant proteins, however, purification of active proteins from these hosts is still challenging. Production of recombinant proteins in plant systems has a number of advantages over microbial and animal systems, including low cost and simplicity of large-scale production [9]. Plants can produce large amounts of heterologous proteins in comparison to yeast and bacterial systems, resulting in reduced cost of production to 0.1% of mammalian cell culture [10]. In addition, folding and assembly of multidomain proteins such as antibodies are possible in plant cells [11]. Some heterologous proteins produced in plants can be directly used without downstream purification processes that significantly decreases the cost of enzyme production [12]. Because of these advantages, plants, as safe and simple bioreactors, have been recently reported as a possible platform for the cost-effective production of recombinant chymosin from native chymosin

gene sequences originated from animals [13,14]. A special advantage of producing recombinant proteins in transgenic plants is that they do not propagate mammalian pathogens [15,16]. This will eliminate the fear of pathogen transmission from animals to humans.

An important fact to be considered in the expression of recombination protein in heterologous hosts is the specific variation in codon usage. This is often assumed as one of the vital factors that have a great effect on protein expression levels [17,18], and therefore, an adaptation of transgene sequence with host codon usage can play an important role in higher expression levels of recombinant protein. It has been shown that applying codon optimization approaches can raise the protein expression level up to 1000-fold [19].

Compartmentalization of recombinant protein can also boost recombinant protein concentration [20]. Transferring recombinant protein to the chloroplasts can protect it from the effects of cytosolic proteases. Also, due to the independence of the chloroplast organelle from other cellular components, it can reduce the toxicity of recombinant protein to the host plants [21]. In addition, regulatory sequence especially selecting the right promoter determines the expression level of a foreign gene in a heterologous host. In this research, a codon-optimized bovine prochymosin gene fused to a chloroplast transit peptide, under control of the strong cauliflower mosaic virus 35S promoter and nopaline synthase terminator, was introduced into tobacco genome using gene gun method. The ultimate goal of this study was to evaluate production of biologically active recombinant chymosin by overexpression of a codon-optimized bovine prochymosin in tobacco plant as a safe, easy to handle and cost-effective system.

MATERIALS AND METHODS

Plant Material

Seeds from tobacco (*Nicotiana tabacum* L. cv. Petite Havana) plants were surface-sterilized with 6% commercial bleach for 5 min, followed by germination and growth on 1/2MS [22] medium containing 30 g/L sucrose. Leaves of three-week-old sterile plants were used as a target tissue for biolistic bombardment.

Vectors Construct

The p35S-TP-CYM expression vector containing a synthetic bovine prochymosin gene was used in transformation experiments (Fig. 1a). To construct this vector, the codon-optimized synthetic prochymosin gene for ideal expression in the nucleus of *Nicotiana tabacum* (Supplementary data 1) was replaced with the GFP gene in p35S-TP-GFP (kindly provided by Prof. Ralph Bock, Max-Planck Institute for Molecular Plant Physiology, Golm, Germany) by means of *Sma*I and *Sac*I restriction sites. The pML74 vector containing the *nptII* selectable marker gene (kindly provided by Prof. Ralph Bock, Max-Planck Institute for Molecular Plant Physiology, Golm, Germany) was used to select stable transgenic plants after co-transformation. In both vectors, transgenes are driven by the CaMV 35S promoter.

Transformation and Regeneration of Transgenic Plants

One day prior to biolistic transformation, young leaves of sterile plants were placed from the abaxial side on an osmotic MS medium containing 120 g/L sucrose. The leaves were then co-transformed with 0.6 µm gold particles coated with both vectors p35S-TP-CYM and pML74 using a Helium-driven biolistic gun (PDS1000He; Bio-Rad) system with a hepta adaptor. After shooting, leaf samples were maintained in dim light for three hours, followed by cutting in 3 mm^3 pieces and transferring on MS medium supplemented with 1 mg/L BAP, 100 µg/L NAA, 30 g/L sucrose and 50 mg/L kanamycin for regeneration and selection of transgenic plants. Regenerated plants were transferred on an antibiotic-containing MS medium complemented with 0.5 g/L GA_3 for boosting plant growth and development in glass vessels. Rooted plants were selected as possible transgenic candidates for further analysis by molecular approaches.

Molecular Analysis of Transgenic Plants

PCR and RT-PCR techniques were used for the analysis of transgene integration and expression in transgenic candidates. For PCR, genomic DNA was isolated from putative transgenic plants by the CTAB method [23]. Selected resistant plants were first screened by PCR analysis using a pair of specific primer for *nptII* selectable marker gene (NPTII-F: 5'-ATG ATT GAA CAA GAT GGA

TTG CAC-3', NPTII-R: 5'- TCA GAA GAA CTC GTC AAG AAG G-3'; Melting temperature 55 °C; Product size 795 bp). Positive lines were further analysed by second PCR experiment using a pair of mature chymosin-specific primers (CYM-F (*NdeI*):5'-AAA CAT ATG GGA GAA GTA GCT TCT GTA C -3', CYM-R (*Xba*I): 5'-AAA TCT AGA TTA AAT AGC TTT AGC TAA TCC TAC-3'; Melting temperature 58 °C; Product size 990 bp). For RT-PCR experiment, total RNA was extracted by RNX-Plus solution (CinnaGen Co.), followed by treatment with DNase I to remove any trace of DNA. The cDNA was prepared from 5 µg of the extracted RNA using SuperScript™ III Reverse Transcriptase, according to the manufacturer's instruction (Thermofisher Scientifics). The above-mentioned pair of mature chymosin gene-specific primer was used to amplify transgene cDNA copies of 990 bp in size. PCR program included a 4-minute stage at 95 °C; 30 cycles, each consisting of 45 s at 95 °C, 45 s at 55 °C and 45 s at 72 °C; and a final extension at 72 °C for 10 min. The housekeeping gene actin was used as a control.

Bioactivity Assay of Recombinant Prochymosin Enzyme

Total soluble protein (TSP) was extracted from RT-PCR-positive transgenic lines. To activate prochymosin, the pH of TSP was adjusted to 2.0 by adding HCl, followed by incubation at 4 °C for 2 h. The pH of TSP was then adjusted to 6-7 pH by 4 M NaOH for milk clotting experiments. To determine milk coagulation, 50 µL of TSP was added to 400 µL fresh milk containing 55 µL of 100 mM of $CaCl_2$, followed by vortexing for a few seconds and incubation at 35 °C with moderate shaking at 70 rpm [14]. This experiment was repeated three times for each transgenic line, and the clotting times were recorded. The data were analyzed by the SPSS software, and mean comparison was performed with Duncan's multiple range test.

RESULTS AND DISCUTION

Chymosin, an aspartate proteinase, generates insoluble para-k-casein by acting on Phe105-Met106 peptide bond presenting in milk k-casein molecules [24], resulting in milk clotting. The native enzyme that is traditionally used in the production of different cheese types, is synthesized in the fourth stomach of suckling calves as preprochymosin precursor protein (381 amino acids). A 16-amino acid signal peptide sequence is then removed to produce inactive prochymosin. The acidic condition of the stomach facilitates cleavage of a 42-amino acid pro-peptide at the N-terminus of this zymogen to generate active chymosin [25]. It has been reported that chymosin has optimal coagulant activity within a pH range of 5-6.9 at 35-40 °C [26]. In this study, we used the pH range of 6-7 at 35 °C.

To date, chymosin from different sources including animals, microbes, and plants is used by the cheese industry (Vallejo J. A. et al. 2008). Due to the limited amount of natural chymosin, recombinant form of this enzyme is the main source of chymosin [27]. Bovine chymosin has high clotting to proteolytic activity ratio, and therefore, is highly suitable for the cheese production industry [5]. Recombinant chymosin has been produced in different systems such as *E. coli* [2,28], *Pichia pastoris* [7,29,30] and plants [13,14]. Among them, plants as a safe bioreactor system can be used for large scale cost-effective production of recombinant chymosin. The production of chymosin in plants can unbind us from severe dependence on livestock, which is one of the main factors involved in global warming [31]. In addition, in contrast to livestock, plants can reduce greenhouse gases such as carbon dioxide by the photosynthesis process. The expression of foreign proteins in eukaryotic heterologous hosts can be increased by optimizing its codons [32] as well as targeting the heterologous protein to the encapsulated organelles such as chloroplasts [33]. Previous studies showed that all three forms of chymosin gene including preprochymosin, prochymosin and chymosin could produce the active form of recombinant chymosin, however, prochymosin form had the highest enzyme activity [34]. In this study, we designed a codon-optimized version of the prochymosin gene for optimal expression in tobacco plants, as a non-food easy to produce plant system. The Codon Adaptation Index (CAI) of our synthetic prochymosin gene for tobacco nuclear genome was 0.929, while this amount is 0.628 for native bovine prochymosin gene, which can lead to higher expression of heterologous protein in transgenic plants [35]. Generally, the ideal range of CAI is around 0.8- 1 [36]. Using *E. coli* expression system, optimization of this index for codons of the bovine

prochymosin gene has been resulted in 70% higher chymosin production in comparison to the natural version [37]. Similar results has been reported in *Kluyveromyces lactis* in another independent study [38].

Here, we used biolistic co-transformation method to integrate the prochymosin construct (Fig. 1a) together with another vector containing *nptII*-selectable marker gene into the tobacco genome. Overall, we obtained one hundred resistant shoots from selecting on kanamycin-containing medium (Fig. 1). To eliminate false-positive resistant lines at primary stages, root formation on antibiotic-containing medium was used as an indicator (Fig. 1d). Selected lines were then analyzed by PCR technique for the presence of selectable marker gene using a pair of *nptII*-specific primers.

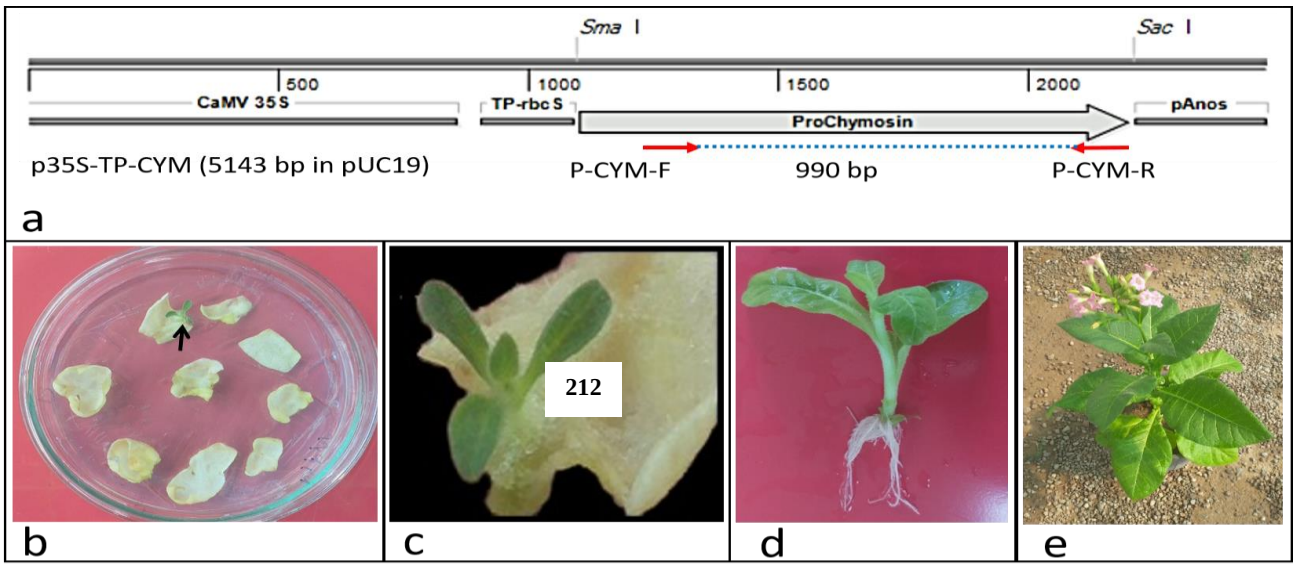

Fig. 1 (**a**) Physical map of the recombinant vector p35S-TP-CYM used for integration of synthetic prochymosin gene into tobacco genome. (b,c) Regeneration of transgenic candidates on selection medium. A sample of regenerated plant is indicated by arrow in part b. The close-up picture of a regenerated shoot on selection medium is presented in part c. (d) Root formation from PCR-positive transgenic lines on selection medium. (e) Growth and development of transgenic plants in greenhouse.

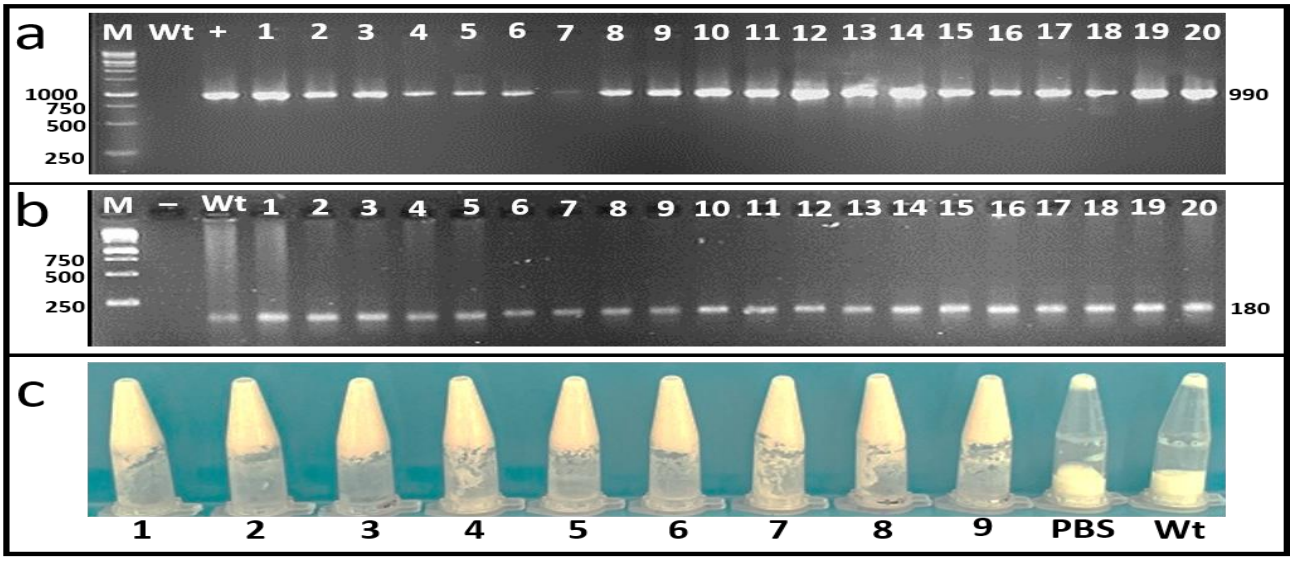

Fig. 2 Agarose gel electrophoresis of RT-PCR products on c-DNA of PCR-positive tobacco prochymosin transgenic candidates. Observation of a 990 bp mature chymosin-specific band demonstrates successful expression of synthetic prochymosin gene in transgenic tobacco plants at transcription level (a). RT-PCR for the housekeeping gene actin was used as a control (b). (c) Milk curdle feature of TSP from a number of transgenic lines (1-9). In contrast to the wild type (Wt), fresh milk clotting was obtained by addition of TSP from all transgenic lines, demonstrating functional chymosin presence in transgenic plant TSP. PBS: blank control.

Ninety-five percent of lines showed *nptII*-specific band, showing that the root formation on selection medium is a powerful indicator of true transformants (data not shown). Transgenic lines showed normal growth and development in the greenhouse condition (Fig. 1e). In the next step, *nptII*-positive transgenic lines were analyzed for the presence of prochymosin gene by PCR technique using a pair of specific primers. In 63% of the lines, a 990 bp mature chymosin-specific band was detected (data not shown). To evaluate prochymosin expression in selected lines, reverse transcription PCR was performed using chymosin-specific primers (Fig. 2a). The housekeeping gene actin was used as a control for proper cDNA synthesis and the absence of genomic DNA contaminations (Fig. 2b).

After confirming with PCR and RT-PCR techniques (Fig. 2a,b), transgenic candidates were tested for the production of functional chymosin. Raw TSP extracted from selected lines was subjected to acidic pH (pH = 2) to activate chymosin enzyme and then it utilized for assessing milk-clotting feature. Prochymosin is reported to be activated by autonomous splicing of pro section in acidic pH [39]. In contrast to the wild type, all tested transgenic lines showed fresh milk precipitation feature (Fig. 2c), however, the coagulation time appeared to be different among them. These results demonstrate the successful production of functional chymosin enzyme in transgenic tobacco plant. In a previous study, native bovine preprochymosin has been integrated into the tobacco genome [14], resulting in successful heterologous protein production. In this experiment, milk-clotting time using TSP was 20.5 to 46.3 min [14]. In our experiments, the milk-clotting time was ranged between 15 (in lines No. 1 and 9 in Fig. 2c) to 60 min. This might be related to the higher expression level in some of the transgenic lines due to the codon optimization and protein targeting into the chloroplast. The transgene position effect, however, can lead to lower expression levels in other transgenic lines [40]. The accumulation of heterologous proteins retained in the cytosol is usually below 0.1% of total soluble proteins [41]. Moreover, the negative redox potential of the cytosolic environment is unsuitable for proper folding of disulfide bond-containing proteins such as chymosin [42]. However, targeting transgene products into chloroplasts would increase its yield. Chloroplasts occupy most of the space in plant leaf cells after vacuoles. Also, in comparison to the vacuoles, chloroplasts have neutral pH that minimizes proteolysis activity [43], which in turn, can result in higher protein accumulation. As an example, post-translational import of heterologous protein into the chloroplasts increased its level up to 11% of total soluble protein [44]. In *Nicotiana benthamiana* Domin, accumulation of human growth factors in the cytosol caused detrimental effects on leaves, while by chloroplast targeting, no side effects was noticed [45]. In consistent with these results, we did not observe any abnormal plants in our study. Expression of functional prochymosin has been also reported in transgenic maize [13], however, there is no report of heterologous expression of mature chymosin in plants. To date, different bovine proteins have been successfully produced in plant systems. Plant-derived recombinant trypsin, as a serine protease, is commercially available in the markets [46]. Aprotinin is another example of bovine-derived enzymes that is heterologously produced in plant systems [47,48]. All to gather, the results show that plants are excellent platforms for the production of bovine-derived enzymes.

Here, we used co-transformation method [49-51] to integrate the prochymosin and the selectable marker gene into the tobacco genome independently. This can be later used for the elimination of marker genes to produce marker-free transgenic lines through segregation in the T1 generation [52].

In conclusion, we produced several tobacco transgenic lines expressing a codon-optimized bovine prochymosin gene. Production of functional chymosin in transgenic plants was confirmed by milk-clotting assay. Our results provide further evidences that plants can be a safe and cost-effective bioreactor for the production of recombinant chymosin.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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SUPPLEMENTARY DATA

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