Production and secretion of recombinant thaumatin in tobacco hairy root cultures

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Production of recombinant proteins in plant cell or organ cultures and their secretion into the plant cell culture medium simplify the purification procedure and increase protein yield. In this study, the sweet-tasting protein thaumatin I was expressed and successfully secreted from tobacco hairy root cultures. The presence of an ER signal peptide appears to be crucial for the secretion of thaumatin: without an ER signal peptide, no thaumatin was detectable in the spent medium, whereas inclusion of the ER signal peptide calreticulin fused to the N terminus of thaumatin led to the secretion of thaumatin into the spent medium of hairy root cultures at concentrations of up to 0.21 mg/L. Extracellular thaumatin levels reached a maximum after 30 days (stationary phase) and the subsequent decline was linked to the rapid increase of proteases in the medium. Significant amounts of thaumatin were trapped in the apoplastic space of the root cells. The addition of polyvinylpyrrolidone and sodium chloride into the culture medium led to an increase of extracellular thaumatin amounts up to 1.4 and 2.63 mg/L, respectively. Thaumatin production compares well with yields from other transgenic plants, so that tobacco hairy roots can be considered an alternative production platform of thaumatin.

Keywords: Agrobacterium rhizogenes · Hairy roots · Recombinant protein · Sweet-tasting protein · Thaumatin

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1 Introduction

Plant cell and tissue cultures are increasingly being used in the production of recombinant proteins for pharmaceutical and industrial purposes. Several advantages of using plant cell cultures for recombinant protein production have been described: plant cells can carry out many post-translational modifications similar to mammalian cells, simplify large-scale production, bear no risks for humans as pathogens, and potentially provide economic benefits due to simple medium and culture conditions [1, 2]. Furthermore, the major advantages of plant cell and tissue cultures in recombinant protein production compared with whole plants are independence from field cultivation conditions and simpler procedures for downstream processing and protein purification, especially when proteins are secreted into the culture medium [2]. Recovery and purification of recombinant proteins from plants usually constitute the majority of production costs, for example, in the case of β-glucuronidase production from transgenic corn seeds about 88% of the expenses are attributed to protein extraction and purification [3]. Therefore, production of recombinant proteins in plant cell and tissue cultures and their secretion into the culture medium is a strategy to simplify the purification procedure thereby reducing production costs.
Hairy root cultures meet the demands of a simple, economic production system: they can be obtained either by infecting transformed plants or explants with *Agrobacterium rhizogenes* or by using genetically modified *A. rhizogenes* harboring binary vectors. Hairy roots can easily be cultured in hormone-free media and are accessible to genetic manipulation. Hairy roots with rapid growth rates can be used as a continuous source of secondary metabolites and valuable recombinant proteins because they can be divided and subcultured several times [4]. Nevertheless, the low level of protein accumulation in plant cells and the instability of secreted proteins in the plant culture medium, which contains a number of hydrolyses and proteases, create new challenges [5, 6]. Therefore, enhancing the protein stability and increasing the protein expression level in plant tissue cultures are key issues for an effective production of recombinant proteins [7].

In this study, we employed tobacco hairy root cultures to produce recombinant thaumatin. The thaumatins, which are a family of sweet-tasting proteins, can be isolated from the fruits of *Thaumatococcus danielli*, which is a herbaceous plant that grows in the rainforests of West Africa. Thaumatins are a mixture of some variant proteins, of which only the two major thaumatins I and II have been studied in detail. Thaumatins I and II each contain a single polypeptide with 207 amino acid residues and have a basic character, as manifested by isoelectric points above 11.5 [8, 9]. The amino acid sequence of thaumatin II differs from that of thaumatin I at five positions (N46K, S63R, K67R, R76Q, and N113D) [10]. The tertiary structure of thaumatin is stabilized by the presence of eight disulfide bridges, which makes it unusually stable with respect to heat and pH [11]. Thaumatins are 100 000 times sweeter than sucrose on a molar basis and are considered a good substitute for common sweeteners in the food industry. However, the limited availability of thaumatin from natural plant sources, due to regional cultivation restrictions, has encouraged alternative production methods of thaumatin. So far, recombinant thaumatins I and II have been expressed in *Escherichia coli* [12–14], *Bacillus subtilis* [15], *Streptomyces lividans* [16], *Aspergillus awamori* [17, 18], *Saccharomyces cerevisiae* [19], and in *Pichia pastoris* [20, 21]. Thaumatin II was also expressed in transgenic potato, tomato, cucumber, strawberry, pear, barley, and tobacco plants [22–28]. In most cases the thaumatin yield has been rather low.

Herein, we report a study on the production of recombinant thaumatin I in tobacco hairy roots. The requirement for eight disulfide bridges in the tertiary structure of thaumatin suggests that it can be targeted to the ER, in which protein disulfide isomerases catalyze the rearrangement of disulfide bonds. The correct disulfide bridge formation may lead to an increase of active thaumatin. For this reason, we investigated the effects of the calreticulin signal sequence on the secretion of thaumatin into the culture medium. Moreover, the effects of medium additives, such as polyvinylpyrrolidone (PVP) and NaCl, on thaumatin recovery were also evaluated.

## 2 Materials and methods

### 2.1 Materials

*E. coli* strain DH5α was used as a host for DNA manipulation and *A. rhizogenes* strain ATCC 15834 (provided by Prof. Dr. W. Alfermann, Düsseldorf, Germany) was used for plant transformation. Gateway® destination vector pK7W2G2D [29] was employed for plant transformation (for details, see http://gateway.ups.ugent.be). Leaves of *T. danielli* were obtained from the Botanical Garden Heidelberg, Heidelberg University.

### 2.2 Cloning of thaumatin I gene and binary vector construction

The roughly 0.62 kb thaumatin I gene was amplified by PCR from *T. danielli* genomic DNA and cloned into pDRIVE (Qiagen). The primers Thau_SacI 5′-GGGGGAGCTCGCCACCTTCGAGA TCGTCAA-3′ and Thau_HindIII_Histag 5′-GGGG AAAGTTTAAATGTTGGTGTGGTATGGGCAT GT-3′ were used for PCR. For easier purification, the thaumatin gene was fused to a poly-histidine tag at the C terminus.

To directly thaumatin to the secretory pathway of the plant cell, the thaumatin gene was N-terminally fused to the calreticulin signal sequence (Z71395). As a control, a vector containing thaumatin without the signal sequence was also constructed (Fig. 1). Binary vectors were established according to the instructions of Gateway® Technology, Invitrogen. The nucleotide sequence of thaumatin in all constructs was confirmed by DNA sequencing using the DYEEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare) and MegaBACE™ 1000 sequencer (GE Healthcare). Sequences were analyzed with BioEdit version 7. The identity of the recombinant protein was unambiguously confirmed by MALDI-TOF analysis.
2.3 Establishment of tobacco-transformed root cultures

Mature leaves from tobacco plants, which were grown aseptically, were cut into 0.5–1 cm² squares in 1/2 Murashige and Skoog (MS) liquid medium [30]. Explants were dried on sterile filter papers and placed on woody plants (WP) solid plates for 2–3 days [31]. About 50 precultured explants were transferred onto a new plate containing 30 mL of an overnight suspension of A. rhizogenes and incubated for 30 min with gentle shaking. The bacteria were poured off and the explants were dried on sterile filter paper and placed on WP solid medium for 2–3 days under low light intensity. After three days of inoculation with bacteria, the explants were placed onto new WP plates containing antibiotics (200 mg/L cloramfen (Sanofi Aventis) and 100 mg/L kanamycin (Sigma)). The explants were subcultured on new WP antibiotic plates every two weeks until hairy root tips appeared. Hairy root tips (4–5 cm) were cut off and then transferred to 250-mL flasks containing 50 mL of WP liquid medium and placed on a shaker at 60 rpm and (24±2)°C for further analysis.

To confirm that hairy root clones were transformed, their genomic DNA was isolated according to a procedure reported by Khanuja et al. [32]. PCR was performed to show the presence of thaumatin and rolC genes and the absence of virC genes. The primers used were 5'–ATGGGCTGAGACGACCTGTGTT-3' and 5'-TATAGCCATGGCAACTTTGCAC-3' for rolC; 5'–ATCATTTGAGCGACT-3' and 5'-AGCTCAAACCTCTTT-3' for virC.

2.4 Time course of growth of tobacco hairy root transformants

Two hairy root transformants (each exerting kanamycin resistance, thaumatin-positive PCR, and a fluorescence signal from vector-encoded green fluorescent protein (GFP)) were selected for growth analysis: pK7WG2D/thau, pK7WG2D/cal/thau (without and with calreticulin signal sequence, respectively). Wild-type hairy roots served as controls. Approximately 0.2–0.25 g fresh weight of 20-day-old wild-type and transgenic clone hairy roots were cultured in 250-mL flasks containing 50 mL WP medium. Hairy root lines were maintained in 250-mL flasks containing 50 mL WP liquid medium and subcultured every three weeks. The time course of growth was determined over a period of 35 days, measuring fresh biomass of hairy root clones every 5 days.

2.5 Protein preparation and immunological detection and quantification of thaumatin

2.5.1 Extracellular protein

The spent medium from each hairy root culture was directly used to determine the concentration of extracellular protein by Bradford microassay and western blot.

2.5.2 Soluble intracellular protein

Approximately 1 g of fresh hairy roots from each clone was homogenized and the total soluble protein (soluble intracellular protein) was extracted by extraction buffer (300 mM NaCl, 50 mM NaH₂PO₄, 0.1% (w/v) Tween-20, 5 mM phenylmethylsulfonyl fluoride (PMSF), pH 8). The protein concentration of the supernatant was determined by Bradford standard assay; the supernatant was diluted to a 1 mg/mL stock for ELISA analysis or stored at -80°C.

2.5.3 ELISA

The total soluble protein was diluted to 40 μg/mL with coating buffer (50 mM Na₂CO₃ and 0.02% (w/v) NaN₃, pH 9.6) and 100 μL of this aliquot were used for the coating of a 96-well microplate (Greiner, Cat. #655077). The plate was blocked with 5% fetal bovine serum in TBS-T (10 mM Tris-Cl, 150 mM NaCl, pH 7.5, 0.05% Tween-20). For immunological
reactions, the plate was initially incubated with 1:10,000 diluted rabbit thauatin antiserum (Biogene) and 1:15,000 diluted goat antirabbit HRP-conjugate. The Supersignal ELISA femto maximum sensitivity substrate (Pierce) was used for detection and the luminescence signal was read in a microplate reader (Tecan safire², Männedorf). For normalization of this assay, the total soluble protein from wild-type hairy roots was used as the diluent for the thauatin standard curve.

2.5.4 Western blotting
Proteins were separated in a 12% SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) using a semidry blotting apparatus (Fisher). After blocking with 5% nonfat dry milk in PBS-T (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄·2H₂O, 1.9 mM KH₂PO₄, 0.05% Tween-20), the membrane was incubated with thauatin antiserum (1:5000) and later with HRP-conjugated goat antirabbit secondary antibody (1:10000). Western Lightning Chemiluminescence Reagent was prepared according to the supplier's instructions (PerkinElmer). The membrane was then documented by using an LAS 3000 Imaging System (Fujifilm). Serial dilutions of thauatin (Sigma) were prepared for each blot. Thauatin band intensities were quantified using ImageJ version 1.42i. The proteolytic activity in the medium of the hairy root cultures was estimated according to the manual of universal protease substrate (Roche Diagnostics, Mannheim).

2.6 Effect of additives
Final concentrations of 1.5% PVP (at day five) or 100 mM NaCl (at day 15) were added to hairy root cultures, respectively. Samples were taken on day 25 of cultivation to measure biomass as well as intracellular and extracellular thauatin contents.

3 Results
3.1 Establishment of transformed hairy root cultures
Approximately 50 explants were infected with A. rhizogenes ATCC15834 harboring one of the vectors, pK7WG2D/thau or pK7WG2D/cal/thau (Fig. 1). A hairy root line transformed with A. rhizogenes ATCC15834 (referred to herein as Nt/ATCC) without plasmid served as a negative control. These explants were selected and transferred to fresh WP selection plates every two weeks until hairy root tips appeared. After three to five subcultures, hairy root transformants were checked for thauatin integration into the genome by PCR amplification using primer pairs specific for thauatin, rolC, and virC: thauatin was present in all of the transgenic clones but not in the wild type, whereas the virC PCR product was absent (data not shown). These results proved that the transgenic cultures were actually transformed with thauatin and were not contaminated with Agrobacterium [33].

The growth curves of the transformed and wild-type hairy root cultures were similar and approached the stationary phase between days 20 and 25 (Fig. 2). Among the investigated lines, pK7WG2D/cal/thau had the highest growth rate and reached a maximal fresh biomass of (5.01±0.75) g/flask. pK7WG2D/thau exhibited the lowest growth rate and reached (4.44±0.25) g/flask on day 20.

The detection of GFP in spent medium (extracellular) and in the total soluble protein isolated from hairy roots (soluble intracellular) was used to monitor recombinant protein synthesis in transgenic plants and to determine variations in recombinant protein expression between different transgenic lines [34]: the high fluorescent intensity of intracellular protein of the three transformants was clearly distinguished from that of non-transformed hairy roots (Fig. 3A). To investigate the effect of an ER signal sequence on protein localization, we transformed hairy roots with GFP fused to the chitinase signal sequence. These cells showed green fluorescence mainly in the apoplastic space and around the nucleus. In transformed cells con-
taining GFP without the ER signal sequence, the green fluorescence is distributed throughout the cytoplasm (Fig. 3B).

### 3.2 Effect of ER signal sequence on thaumatin expression

Aiming to secrete recombinant thaumatin into the culture medium of tobacco hairy roots, we fused the calreticulin ER signal peptide to the N terminus of thaumatin in the binary vector pK7WG2D.

Time courses of intra- and extracellular thaumatin production from three hairy root transformants were recorded every 5 days for 35 days (Fig. 4). The intracellular thaumatin content was determined by ELISA, whereas the extracellular thaumatin content was detected by western blotting and estimated by band density using ImageJ. Western blotting was chosen to estimate extracellular thaumatin levels instead of ELISA because of difficulties in establishing standard curves: abundant phenolic compounds present in tobacco cell media may have interfered with the chemiluminescence reaction [35, 36]. Time courses of intracellular thaumatin expression from two transformed hairy root clones are shown in Fig. 4A. A comparison of recombinant thaumatin expression between transformants revealed that pK7WG2D/thau expressed thaumatin in the range of 0.12–0.13% of the total intracellular soluble protein, whereas pK7WG2D/cal/thau expressed 0.93–1.44%. The expression of thaumatin from pK7WG2D/cal/thau increased from day 10 to 30 and decreased after 30 days. Time courses for extracellular thaumatin from two transformed hairy root clones are shown in Fig. 4B. The pK7WG2D/cal/thau transformant reached a maximum of 0.208 mg/L after 25 days, whereas no extracellular thaumatin was detected in the case of pK7WG2D/thau. The loss of secreted thaumatin from the medium of the pK7WG2D/cal/thau culture after 30 days is linked to the rapid increase of proteases in the medium (Fig. 4C).

To further confirm these results, 4 µg of total protein obtained from cell extracts and 100 µL of spent medium from 20-day-old transformed hairy roots were used for western blotting, along with the Nt/ATCC negative control (Fig. 4D). Immunological detection revealed a thaumatin band in both the cell extract and spent medium of the pK7WG2D/cal/thau transformant, whereas no thaumatin band was observed from pK7WG2D/thau and Nt/ATCC. The molecular weight of the recombinant thaumatin was slightly higher than that of standard thaumatin due to the attachment of a histidine tag to recombinant thaumatin. Furthermore, an additional band of approximately 20 kDa was detected in western blots of both wild-type and transformant cell extracts that might be a result of abundant antibodies in the anti-thaumatin serum.

### 3.3 Effect of NaCl and PVP on thaumatin recovery

To increase extracellular thaumatin, NaCl was added at 100 mM final concentration to hairy root culture pK7WG2D/cal/thau on day 15. Figure 5A shows a 12.6-fold increase of extracellular thaumatin to a concentration of 2.62 mg/L, whereas the concentration of intracellular thaumatin does not appear to be affected by the addition of NaCl.

A similar result was obtained when 1.5 g/L PVP were added at day five of hairy root cultures. The concentration of extracellular thaumatin increased to 1.4 mg/L (a 6.7-fold increase relative to the normal culture medium). Intracellular thaumatin was slightly reduced if it was affected at all. Western blotting (Fig. 5C) clearly revealed extracellular
thaumatin in both NaCl and PVP treatments, whereas a very slight band was observed from the control. The addition of NaCl (at 100 mM) and PVP (at 1.5 g/L) did not affect cell growth significantly; however, slightly lower fresh weights with PVP and NaCl treatments were observed (Fig. 5B).

4 Discussion

Herein we report, for the first time, the production and secretion of recombinant thaumatin in hairy root culture. In this study, two hairy root lines were established that expressed either thaumatin only or thaumatin N-terminally fused to the calreticulin ER signal sequence. A significantly higher thaumatin production in hairy root transformants was observed in the presence of the ER signal peptide. The expression level of recombinant thaumatin was about 12-fold higher in total soluble protein when the signal sequence was present. This is directly associated with the requirement for eight disulfide bridges for the correct folding of the three-dimensional structure of thaumatin. Theoretically, owing to the signal sequence, thaumatin enters the ER, where it starts to undergo post-translational processing. As reviewed by Wilkinson and Gilbert [37], disulfide bridges are mostly formed in the ER, but not in the cytosol of eukaryotic cells. This is due not only to the oxidized state of the ER, providing the basis for disulfide bond formation, but also to the abundance of protein disulfide isomerase in the ER, which catalyzes disulfide bridge formation [37]. Moreover, protein targeting to the ER increases the protein yield by correct folding, which prevents the degradation of proteins by abundant proteases in the cytosol [38]. Proteases tend to degrade abnormal or incorrectly processed proteins [7]. In this study, the detection of intracellular thaumatin by ELISA, but concealment by western blot in the case of the transformant lacking the signal sequence (pK7WG2D/
Figure 5. Effect of PVP and NaCl treatment on thaumatin yield. Hairy roots transformed with pK7WG2D/cal/thau were treated with 1.5 g/L PVP or 100 mM NaCl, respectively. Fresh weights, as well as extracellular and intracellular thaumatin contents, were determined at day 25. (A) Extra- and intracellular thaumatin; secreted extracellular thaumatin is shown as µg/mL of spent medium (■); intracellular thaumatin is shown as µg/10 µg total soluble protein of cell biomass (■). (B) Fresh weight of hairy roots. In A and B, the mean and standard deviation for three independent experiments per treatment are indicated. (C) Western blot of spent medium: 50 µL of spent medium with or without PVP or NaCl addition. For size comparison, 10 ng of thaumatin 4 (Sigma) and a prestained size marker (Peqlab) were used. The western blot shows representative data of three experiments.

Thaumatin, could be due to retention of incomplete or misfolded thaumatin in the cytosol. This idea is supported by earlier findings that partially degraded protein fragments can be detected in ELISA assays [7].

Extracellular thaumatin of pK7WG2D/cal/thau hairy roots reached a maximum concentration during the stationary growth phase after 25 days. The following decline of extracellular thaumatin is linked to the rapid increase of proteases in the medium after day 25 (Fig. 4C). The loss of secreted thaumatin from the medium of hairy root is in good agreement with previous results, showing that proteases are one factor responsible for the loss of extracellular proteins [6, 7, 39]; it is also reported that the surface adsorption of culture flasks may contribute to the loss of extracellular protein and that the simplicity of plant culture medium does not support the stability of proteins [40]. Intracellular thaumatin levels were higher in pK7WG2D/cal/thau than those in pK7WG2D/thau hairy root transformants. The time course of intracellular thaumatin levels in both these transformants (Fig. 4) revealed an increase during the first 30 days of culture. The maximum production of intracellular thaumatin from pK7WG2D/cal/thau (1.44% total soluble protein) was higher than the yields reported in pear, cucumber, tomato, and strawberry [23, 25, 26, 41], but lower than the values reported in barley [27] for thaumatin production in transgenic plants.

The hairy root cultures show a higher proportion of thaumatin in total soluble protein than in the spent medium. A likely explanation for this is that proteins larger than 20–30 kDa (recombinant thaumatin ~23 kDa) tend to be retained in the apoplast [42]. For example, Borisjuk et al. [43] showed that the use of the calreticulin signal sequence fused to GFP led to higher GFP accumulation in the apoplast than in the hydroponic medium and the cellular protein fractions. Moreover, because the pI value of 11.5 for thaumatin is extremely basic, thaumatin is possibly trapped by ionic forces in the apoplastic space of the cell wall where conditions range from pH 5 to 6.5 [44].

The use of medium additives, such as sodium chloride and PVP to improve protein accumulation in plant cell cultures has been observed in several studies [45–49]. However, to the best of our knowledge, this is the first time that sodium chloride has been used to enhance the level of secreted protein in hairy root cultures. Sodium chloride addition resulted in an increase not only of secreted thaumatin (12.6-fold), but also of total supernatant protein (two-fold) in spent medium (data not shown). This is in contrast to previous results, for which the
highest production of recombinant protein with the addition of sodium chloride in plant cell suspension exceeded the control by only about 2.3-fold [48]. The significant increase in secreted thaumatin might be due to repulsive ion forces between apoplastic thaumatin and the added ions. Besides, the high level of supernatant proteins in spent medium after salt addition may act as an alternative substrate for protease activity [47, 50]. The addition of PVP as a foreign-protein stabilizer [50], also had a positive effect on the secretion of thaumatin (6.7-fold). This result is in good agreement with previous studies on foreign-protein stability in plant tissue cultures [6, 39, 46, 48, 50].

5 Conclusions

Our experiments show that hairy root cultures of *Nicotiana* provide a suitable system to produce recombinant proteins and to secrete them into the culture medium. More experiments are needed to enhance the secretion into the medium and to prevent protein degradation.

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6 References


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