



Agrobacterium Rhizogenes-Mediated Transformation of Antifreeze Protein in Tobacco Hairy Roots

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Abstract

Introduction: Antifreeze proteins (AFPs) decrease the freezing point, not the melting point, of aqueous solutions through inhibition of ice crystal growth by an adsorption-inhibition mechanism. AFPs have many applications in cryopreservation. Nevertheless, low yield and high extraction cost of extracting AFPs from natural sources limit their wide applications. To overcome this limitation, here we genetically transformed tobacco hairy roots mediated by *Agrobacterium rhizogenes*.

Materials and Methods: Recombinant vector was transformed into *Agrobacterium rhizogenes* strain 15834 by heat-shock method. *Nicotiana tabacum* leaf discs were co-cultured with recombinant *Agrobacterium rhizogenes* under vacuum condition. PCR was used to examine whether transgenic lines were free from *A. rhizogenes* and to confirm integration of AFP gene. Western blotting was performed to confirm AFP expression.

Results: Using this method, we achieved a high transformation efficiency of 86.7%. Molecular analysis confirmed the integration of the AFP gene and the absence of *A. rhizogenes* contamination in the hairy root lines. Western blotting confirmed a high level of AFP expression, with a molecular mass of approximately 20 kDa. After 30 days of cultivation in flasks, a hairy root biomass of approximately 4.9 g was obtained.

Conclusions: Tobacco hairy root expression platform offers a fast, low-cost, and efficient system for heterologous production of antifreeze proteins and should be considered to employ in industrial scales in the future.

Keywords: Antifreeze Protein, Heterologous Expression, *Agrobacterium rhizogenes*, Hairy Root, *Nicotiana tabacum*

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Introduction

Antifreeze proteins (AFPs), also known as thermal hysteresis proteins (THPs), ice-binding proteins (IBPs), and ice structuring proteins (ISPs) are a unique class of proteins which recognize, interact with, and inhibit the growth of ice crystals in cold-adapted ectothermic organisms. AFPs inhibit ice growth by adsorbing particular facets of ice crystals at temperatures below zero.¹ AFPs are abundant in fishes, insects, plants, bacteria and fungi.² AFPs share physical properties, while their structures and sources differ. Thermal hysteresis (TH) and ice recrystallization inhibition (IRI) are known as the activities of AFPs.³ TH interrupts the thermodynamic melt/freezing point of a solution, whereas IRI alters the shape or structure of ice crystals and manipulates the growth rate of ice crystals. Both activities of AFP in cold-adapted organisms are responsible for reduction in tissue damage.^{4,5}

AFP genes have been identified in many organisms, including cold-adapted insects, plants, and fish. An interesting idea is to insert an AFP gene in another organism's genome to increase their survival in low or subzero temperatures.⁶ This could potentially expand the

geographic growing regions crops and animals. Some efforts have been taken to apply AFPs to aquaculture and agriculture, yet these methods are not applicable for production.⁷

The potential of AFPs in medical and industrial applications has generated significant interest, particularly where low-temperature storage is essential and ice crystallization must be prevented. In medicine, AFPs are commonly applied in cryopreservation and cryosurgery. Utilizing AFPs as chemotherapy agent and in organ transplantation in particular kidney is under extensive investigation.⁸ In the food Industry, AFPs used to prevent the deterioration of food products like ice cream, increase the shelf-life of bakery products.⁹ AFPs inhibit undesirable changes in the quality of food caused by the loss of nutrients or cellular damage.^{10,11}

Recombinant protein expression in bacteria and yeasts provides the opportunity for industrial-scale production of AFPs, nevertheless, required posttranslational modifications limited their use.¹² Recently, several studies have reported the heterologous production of recombinant AFPs in yeast

hosts such as *Pichia pastoris* (*Komagataella phaffii*), with preserved ice recrystallization inhibition activity and cryoprotective effects in food and cell-freezing applications.^{13,14} Plant-based expression system is a suitable alternative platform to produce AFPs. Several studies have reported the expression of AFPs in whole plants, such as strawberry¹⁵ and tomato,^{16,17} primarily to enhance their cold tolerance. Hair roots offer a more contained and controllable system compared to whole-plant cultivation. Post translational modification like glycosylation, appropriate folding, and disulfide bond formation of recombinant proteins, a requirement for their biological activity, are easily provided by plants.^{18,19} Hair roots (HRs) syndrome is a pathological condition induced by *Agrobacterium rhizogenes* infection which characterized by excessive proliferation of branching roots. HRs is interesting alternatives as expression platforms of mass production of recombinant proteins. In the present paper, recombinant expression of AFP in tobacco hairy root is investigated. The integrity of AFP gene and its expression in HRs was confirmed by PCR and Western blotting respectively.

While various platforms exist for producing recombinant proteins, each has its limitations. Bacterial and yeast systems often struggle with the post-translational modifications required for complex protein function. Plant-based systems, particularly hairy root cultures, offer a compelling alternative due to their genetic stability, low maintenance costs, and ability to perform eukaryotic post-translational modifications. Given the high demand for AFPs and the need for an efficient production method, this study investigates the potential of tobacco hairy roots as a novel expression platform. Specifically, we aimed to establish transgenic hairy root lines expressing a winter flounder AFP, confirm the integration and expression of the transgene, and evaluate

the biomass accumulation of the cultured roots.

Materials and Methods

Plant Materials

The seeds of *N. tabacum* L. cv. Xanthi were obtained from the Thirtash Research and Education Center, Behshahr, Mazandaran, Iran. The surface of the seeds were sterilized by immersing in 70% (v/v) ethanol 30 s, followed by treatment with 2% sodium hypochlorite: Triton X-100: sterile distilled water (30:1:70) for 20 min. The seeds were further rinsed five times by sterile distilled water. The sterilized seeds were placed onto solid Murashige and Skoog (MS) medium (3 % sucrose, 0.6% agar, pH ~ 5.7)¹⁷ to grow at 25 °C, 30 days with a 16/8-h light/dark photoperiod.

Bacterial Strains, Plasmid and Media

Agrobacterium rhizogenes strain 15834 was used as cloning host. Root inducing (Ri) plasmids, pBI121, (Qiagen, Hilden, Germany) was used as the cloning vector containing the kanamycin resistance gene. The AFP structure used in the present study is as follow: a signal peptide to secret the protein into the apoplastic space originated from Acidic endochitinase Q (GenBank accession number P17514), the *Pseudopleuronectes americanus* (Winter flounder) antifreeze protein sequence (GenBank accession number B1P0S1) and histidine tag (Figure 1). The recombinant AFP construct was inserted between CaMV 35S promoter and NOS terminator. The synthetic gene, codon-optimized for tobacco, was synthesized and cloned into the pBI121 vector between the *Xba*I and *Sac*I restriction sites, replacing the GUS reporter gene. The final construct, pBI121-AFP, was verified by restriction digestion and DNA sequencing before being transformed into *A. rhizogenes*.



Figure 1. AFP Construct which Includes the Signal Peptide of Acidic Endochitinase Q (GenBank accession number P17514), the *Pseudopleuronectes americanus* (Winter flounder) Antifreeze Protein Sequence (GenBank accession number B1P0S1) and Histidine Tag Inserted between CaMV 35S Promoter and NOS Terminator at *Xba*I and *Sac*I Restriction Sites.

The sterilized seeds of *N. tabacum* L. cv. Xanthi were placed onto solid Murashige and Skoog (MS) medium (3% sucrose, 0.6% agar, pH 5.7)²⁰ and incubated in a germinator at 25 °C with a 16/8-h light/dark photoperiod (3000 lux light intensity). After 30 days, the resulting plantlets were used as a source of sterile leaf explants. For further propagation and maintenance, the axenic plant cultures were sub-cultured every two months on fresh MS medium. Leaves with a diameter of 1-2 centimeters from these cultures were

selected for *Agrobacterium*-mediated transformation.

Agrobacterium rhizogenes-mediated Transformation and Hairy Root Establishment

Heterologous plasmid was transformed into *A. rhizogenes* strain 15834 by heat-shock method. The transformed *A. rhizogenes* strain 15834 was grown at 27 °C in Yeast Extract-Peptone (YEP) medium (1% yeast extract, 1% peptone, 0.5 % NaCl, pH ~ 7.0) supplemented with 50 µg/ml

kanamycin, and YEP plates prepared and solidified with 2% agar. Previous to the *A. rhizogenes*-mediated transformation, the recombinant bacteria were cultured at 27 °C and collected at the OD_{600 nm} of 0.8 and resuspended in Murashig and Skoog salts without the addition of sugar. Acetosyringone was added at the final concentration of 100 µM and bacterial suspension was kept at room temperature under dark condition for 1 to 3 h prior to vacuum infiltration. *N. tabacum* L. cv. Xanthi leaf discs (2 cm²) were co-cultured with recombinant *A. rhizogenes* harboring the PBI 121-AFP vector for 10 min. The suspension containing the leaf explants was then placed in a vacuum desiccator connected to a vacuum pump. A vacuum of 100 mBar was applied for 10 minutes, with gentle shaking of the desiccator halfway through to ensure even infiltration of the bacterial suspension into the leaf tissue; then the leaf explants were placed on sterile filter papers to dry and further transferred onto solid MS medium. After 3 days the leaf explants were transferred onto solid MS medium supplemented with antibiotics (400 µg/ml cefotaxime and 50 µg/ml kanamycin) for 4 weeks in the dark. Then, leaf explants were transferred to fresh MS medium supplemented with antibiotics (400 µg/ml cefotaxime and 50 µg/ml kanamycin), incubated at 25 °C under a 16/8 h light/ dark photoperiod for 14 days to study the root induction potential of *A. rhizogenes* strain 15834. Hairy roots were further subcultured every 2 weeks

and maintained in MS medium supplemented with antibiotics (400 µg/ml cefotaxime and 50 µg/ml kanamycin) at 25 °C in the dark. Wild-type hairy root (Wt) line obtained by infection of wild non-transformed *A. rhizogenes* to *N. tabacum* as described above.

Genomic DNA Extraction and Confirmation of Transformation

For PCR analysis, seven putatively transformed HR lines (T1–T7) were selected, representing independent biological transformation events. DNA extraction and PCR were performed once for each of these seven lines.²¹

Polymerase Chain Reaction (PCR)

All primers designed for PCR are listed in Table 1. The VirD specific primers were used to confirm *A. rhizogenes* free HR establishment. AFP specific primers were used to confirm the integration of AFP gene into transformed HR DNA designed and analyzed by Oligo Analyzer - 1.0.3. rolC specific primers adopted from Lee et al.²² were used to confirm transformed nature of established HR cultures.

The resulted PCR products were analyzed using 1% agarose gel electrophoresis applying Tris-acetic acid-EDTA (TAE) buffer, pH 8.2 for 50 min, 50 V. The gel was stained with 0.5 µg/ml ethidium bromide and visualized by UV light and photographed for analysis. 1 kb DNA ladder was used in these set of experiments.

Table 1. Primer Sequence Designed for PCR

Name	Annealing temperature (°C)	Sequence (5'→3')	Product length (bp)
F-virD	59	TCAGCATGGAGCAACCGTC	430
R-virD	57	ATGTCGCAAGGCAGTAAGC	
F-AFP	59	CGGCTCTCCAATGGCTCTTT	665
R-AFP	60	AGTGGTGATGGTGATGATGTGG	
F-rolC	56	ATGGCTGAAGACGACCTGTGTT	543
R-rolC	56	TAAGCCGATTGCAAACCTTGCTC	

Total Protein Preparation

Hairy root tissue (100 mg) was ground to a powder under liquid nitrogen with mortar and pestle and suspended in 50 mM phosphate buffer (pH ~ 7), vortexed vigorously for 1 min, centrifuged at ~ 10,000 xg for 20 min at 4 °C. Supernatants were filtered using 0.45 µm membranes. Bradford assay was performed to determine the total protein concentration using bovine serum albumin (BSA) as the standard.²³

Western Blot Analysis of the Recombinant Antifreeze Protein 1

For Western blotting, nine independent transformed HR clones (T1-T6 and T8-T10) were analyzed to assess the consistency of protein expression across different biological replicates. The protein samples consisted of 15 ng of total protein in SDS sample buffer, supplemented with 5% β-mercaptoethanol. Western blotting was performed according

to standard protocols.²⁴ Briefly, the protein samples were loaded onto a 12% SDS-polyacrylamide gel, electrophoresed and transferred to a pre-wet (25 mM Tris, 192 mM glycine and 20% methanol) nitrocellulose membrane, utilizing a mini trans-blot transfer cell and pre-cooled transfer buffer at 4 °C, 100 V, 2 h. Immunodetection using an antibody was performed according to the manufacturer's instructions. Anti-His tag primary antibody (Sigma-Aldrich, product No. A7058) and pyclonal antibody conjugated with HRP were used to identify and visualize the AFP.

Results

Recombinant T-DNA containing AFP was transferred into tobacco HRs by *A. rhizogenes*-mediated transformation. Prior to plant transformation, the integrity of the pBI121-AFP plasmid in the *A. rhizogenes* strain 15834 was confirmed by colony PCR using the AFP-specific primers (Table 1), which amplified the expected 665 bp fragment

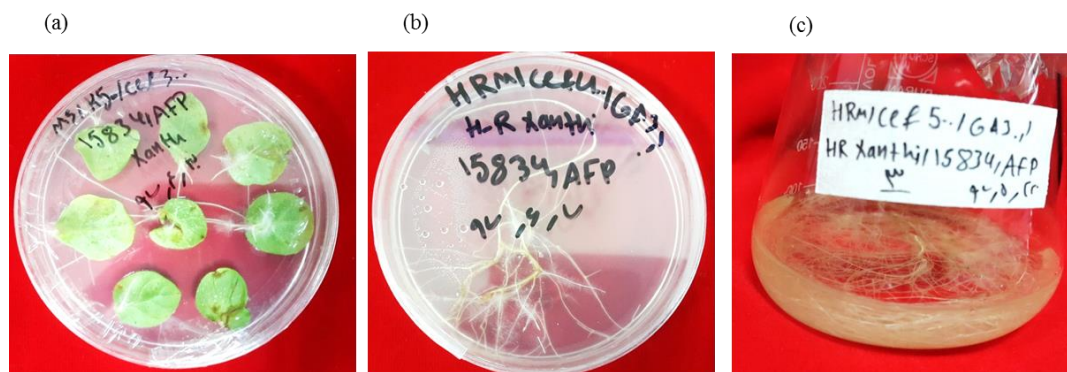


Figure 2. (a) Hairy Roots Appeared in Leaf Explants after 8 Days, in Infection Site in Solid Medium, (b) Development of a Line of Hairy Roots in the Solid Medium and (c) Growing Hairy Roots in Erlenmeyer Flask in a Liquid Culture Medium.

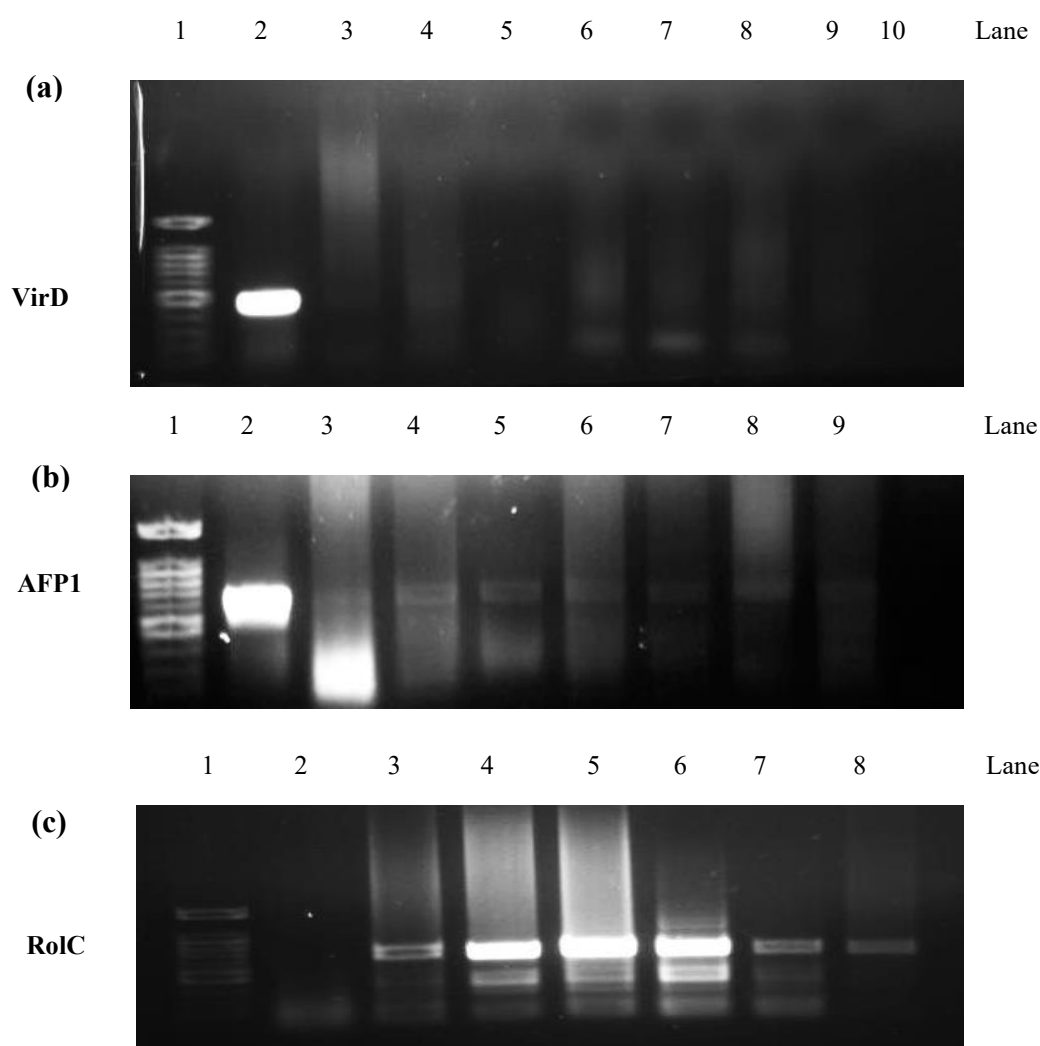


Figure 3. Molecular Evaluation of Transformed HRs (a) *virD* Primers were Used to detect *A. rhizogenes* contamination; Lane 1: DNA ladder, Lane 2: *A. rhizogenes*, Lane 3-9: random transformed HRs (T1-T7). (b) primers were used to confirm AFP1 transformation in to transformed HR; Lane 1: DNA ladder, Lane 2: pBI21-AFP1 (positive control), Lane 3-9: random transformed HRs (T1-T7). (c) RolC primers were used to confirm the integrity of transformed HR genome; Lane 1: DNA ladder, Lane 2: wild type HR, Lane 3-8: random transformed HRs (T1-T6).

(data not shown). Non-transformed *A. rhizogenes* was used to generate the wild type (non-recombinant HRs) as controls. Root initials in leaf explants were observed early at the

infection sites, within 4-10 days and obtained in more than 86% of explant with an average of 3 roots per explant (Figure 2). Out of 30 inoculated leaf discs (2 cm²), 26 discs

produced HRs, and therefore, the transformation efficiency was 86.7%. The integration of the PBI 121-AFP DNA fragment was confirmed by PCR. Molecular studies were performed on seven of well-developed HRs clones (T1–T7) and wild type HRs as negative control. None showed any trace of *A. rhizogenes* contamination when VirD primers (Table 1) were used in PCR amplification (Figure 3a). In order to investigate the integrity of AFP gene in HR genome, transformed HRs were sub cultured in a hormone-free culture medium, 3 times. Then, seven transformed clones were selected and subjected to PCR using AFP and rolC specific primers (Table 1). As expected a PCR fragment with approximately 600 bp length corresponded with the AFP and rolC genes were visualized (Figure 3b, c). These data demonstrate the successful transformation and integration of AFP into HR genome and transformed

HRs were not contaminated with *A. rhizogenes*. Transformed HRs were grown in a hormone-free medium in an Erlenmeyer flask for 30 days to reach a mass of approximately 4.9 g.

The AFP expression in transformed HRs was confirmed by western blotting (Figure 4). Total protein was extracted from nine independent HR clones. As shown by Ponceau S staining of the membrane (Figure 4A), equal amounts of total protein were loaded for all samples, serving as a loading control. Immunodetection with an anti-His tag antibody revealed a distinct band of approximately 20 kDa, corresponding to the expected size of the AFP, in all nine transformed clones (Figure 4B, lanes 1-6, 8-10). No such band was detected in the wild-type HR control (Figure 4B, lane 7), confirming the specific expression of the recombinant AFP in the transgenic lines.

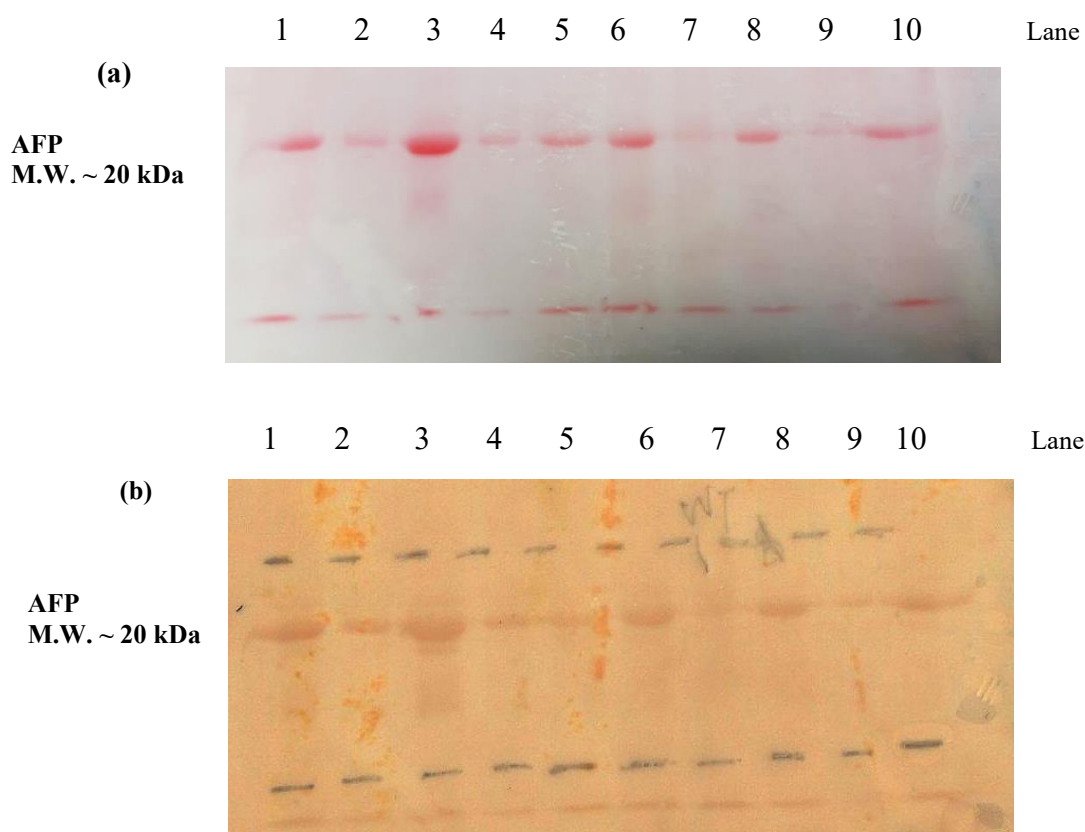


Figure 4. (a) Ponceau S Staining of Expressed APF Blotting in Transformed HRs; Lane 1-6 random transformed HRs (T1-T6), Lane 7: wild type HR, Lane 8-10: random transformed HRs (T7-T9). (b) western blotting of the expressed AFP in transformed HRs; Lane 1-6 random transformed HRs (T1-T6), Lane 7: wild type HR, Lane 8-10: random transformed HRs (T7-T9).

Discussion

Plant tissues and plant cell-based platform represent an outstanding system for heterologous expression of proteins and are growing rapidly. Among cultured plant tissues hairy roots (HRs) have been widely used to produce heterologous. HR-based production platforms offer several advantages including post-translational modifications, low production

costs, lower risk of contamination by viruses, pathogens and toxins, long-term transgene stability, production under sterile contained conditions and high quality. Secretion of the recombinant protein into the culture medium simplifies the recovery and purification of products.^{25,26} For these reasons, a wide range from vaccines to antibodies has been produced in HRs.²⁷⁻³¹ This aligns with the findings of Pham

et al.,³² who successfully produced recombinant thaumatin in tobacco hairy roots, and Moghadam et al.,³³ who expressed the MAP30 protein in the same system, highlighting the versatility of tobacco HRs for producing diverse heterologous proteins. Similarly, Gurusamy et al.³⁴ reported the production of biologically active recombinant human erythropoietin in tobacco hairy root cultures.

In the present paper, an agropine type of *A. rhizogenes* ATCC 15834 was utilized for successful development of hairy roots from leaves explants of tobacco. This strain was used to establish hairy root in tobacco in other studies as well.^{30,32,33,35} HR formation is triggered by bacterial attachment to plant cells, further, integration and expression of T-DNA in plant genome. In line with previous reports,^{36,37} we observed that these neoplastic HRs generally produced massive adventitious roots with lateral branches that grow on hormone-free medium.

rol genes encode proteins responsible for hairy root formation in *A. rhizogenes*. In general, transgenic HRs are subjected to PCR with specific primers for amplification of *rolC* and *VirD* genes. This helps to confirm axenic and transform nature of HRs. In accordance with other studies,^{38,39} here, we demonstrated that HRs were genetically transformed, as shown by *rolC* amplification, and free of *A. rhizogenes*, as no *VirD* amplification product was obtained. (Figure 3).

The growth of the transformed HRs was assessed over 30 days in liquid culture. After this period, the average fresh weight of the transgenic HR lines was approximately 4.9 g per flask. In comparison, wild-type HRs, generated by transformation with the non-recombinant *A. rhizogenes* strain, reached an average fresh weight of 5.2 g under the same culture conditions, indicating that the integration and expression of the AFP gene did not significantly impair hairy root growth.

Heterologous expression of AFPs in crops is reported in many studies, even though the applications of AFP in food are mainly based on extracts instead of genetic modification. Khammuang et al. transformed modified antifreeze protein gene in strawberry mediated by *Agrobacterium*.¹⁵ Heterologous expression of antifreeze protein originated from *Lolium perenne* and carrot confers chilling tolerance in transgenic tomato plants.^{16,17} Insect (*Microdera puntipennis dzungarica*) antifreeze protein expression in tobacco enhanced its cold tolerance.⁴⁰ Unlike these whole-plant approaches, our study focuses on using hairy roots as a dedicated production platform, which allows for protein accumulation under sterile, controlled conditions without the need for whole-plant regeneration and growth in soil.

AFPs are expressed in many platforms including bacteria and yeasts,⁴⁰⁻⁴² While microbial systems like *E. coli*⁴⁰ and *P. pastoris*⁴³ are efficient for producing simple AFPs, the hairy root system offers a distinct advantage for proteins requiring complex eukaryotic post-translational modifications, which can be critical for stability and function.^{18,19} Consistent with

this, recent studies have continued to rely on microbial hosts for the production of recombinant AFPs and ice-binding proteins, including *Pichia pastoris* (*Komagataella phaffii*) for *Glaciozyma antarctica* and snow flea AFPs,^{13,14} *Bacillus subtilis* and *Escherichia coli* for radish ice-binding proteins,⁴⁴ and *E. coli* for a hyperactive DUF3494 ice-binding domain used as a purification tag.⁴⁵ Protein engineering of recombinant snow flea AFP has also yielded variants with enhanced thermal hysteresis and improved cell cryopreservation outcomes,⁴⁶ suggesting that sequence engineering could be combined with plant-based production in future work. The yield of 4.9 g of biomass per flask after 30 days is comparable to other recombinant protein studies in HRs,^{32,33} suggesting that AFP expression does not impose a major metabolic burden on the roots. However, to the best of our knowledge, this is the first report on the heterologous expression of an antifreeze protein in tobacco hairy roots. This finding reveals the excellent and previously unexplored potential of the tobacco hairy root system for the production of AFPs, offering a novel plant-based alternative to existing methods. More investigation is needed to scale up the production of AFPs in transformed hairy roots of tobacco.

While our results at the flask scale are promising, scaling up production to an industrial level presents several challenges that warrant further investigation. The stability of the recombinant AFP within the hairy root biomass and in the culture medium over extended cultivation periods needs to be assessed. Furthermore, developing an efficient and cost-effective protein extraction and purification strategy from root tissue will be critical for commercial viability. The use of bioreactors for large-scale hairy root cultivation also requires optimization of parameters such as aeration, nutrient supply, and shear stress to maximize biomass and protein yield.^{25,26} Future studies focusing on these scale-up parameters will be essential to translate this laboratory-scale success into an industrial process.

Conclusion

In the present study, our findings showed that tobacco hairy root expression system can be employed for heterologous production of antifreeze proteins. Therefore, hairy root expression system is an excellent fast, low cost and easy platforms for expression of AFPs in laboratory and should be considered to employ in industrial scales in the future.

Authors' Contributions

Formal Analysis: MS; Data Curation: MK and SJD; Project Administration: SJD; Supervision: SJD; Writing original draft: MS; Writing – review and editing: SJD. All authors approved the final version of the manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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