Heterologous production of Hyaluronic Acid in *Nicotiana tabacum* hairy roots expressing a human Hyaluronan synthase 2

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Abstract

Hyaluronic acid (HA), a unique polysaccharide with excellent physicochemical properties, is broadly used in pharmaceutical, biomedical, and cosmetic fields. It is widely present in all vertebrates, certain bacterial strains, and even viruses while it is not found in plants, fungi, and insects. HA is naturally synthesized by a class of integral membrane proteins called Hyaluronic acid synthase (HAS). Thus far, industrial production of HA is carried out based on either extraction from animal sources or large-scale microbial fermentation. The major drawbacks to using these systems are contamination with pathogens and microbial toxins. Recently, the production of HA through recombinant systems has received considerable attention. Plants are eco-friendly ideal expression systems for biopharmaceuticals production. In this research, the optimized human hyaluronic acid synthase2 (hHAS2) sequence was transformed into *Nicotiana tabacum* using *Agrobacterium rhizogenes*. The highest hHAS2 production in the tobacco hairy roots was 65.72 ng/kg (wet weight). The extracted HA was verified and quantified by the HA ELISA kit. The DPPH radical scavenging activity of HA with the highest concentration of 0.56 g/kg (wet weight) showed the maximum activity of 46%. Gel Permeation Chromatography (GPC) analyses revealed the high molecular weight HA with about > 0.8 MDa.

Introduction

Hyaluronic acid (HA) (also called Hyaluronan or Hyaluronate), a linear polyanionic mucopolysaccharide, is classified as a Glycosaminoglycan (GAG) ¹. HA is composed of repeating disaccharide subunits of N-acetyl-D-glucosamine and D-glucuronic acid connected by β -1,3-glycosidic bonds, in a way that each repeating disaccharide unit is connected to the other by β -1,4- glycosidic ². The molecular weights of HA from different sources is between 10^4 and 10^7 Da ³.

Hyaluronan is a very interesting molecule famous for its unique Physico-chemical properties. Due to its hydrophilic chemical nature, in the presence of water, it can be expanded up to 1000 times in volume and consequently form the viscous hydrated gel matrices ¹. HA extensively presents in nature for instance in vertebrates ⁴, the capsular component of certain bacteria such as *Streptococcus* sp. and *Staphylococcus* sp. ⁵, the green algae *Chlorella* sp. affected by the *Chlorovirus* ^{4,6}, yeasts, such as *Cryptococcus neoformans* ⁴ and mollusks ⁷. However, it is not found in fungi, plants, and insects ⁸. Almost 50% of the HA content in the human body exists in the skin ⁹. Synovial joint fluid, eye vitreous body, cartilage tissue, and umbilical cord also contain large amounts of hyaluronan. HA can provide a broad range of medical, pharmaceutical, cosmetic, and dietary applications ^{8,10}.

Hyaluronan is synthesized by an integral membrane protein known as Hyaluronic acid synthase (HAS) ¹¹. Hyaluronan synthase enzyme transfers each glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) moieties and catalyzes two distinct intracellular glycosyltransferase reactions ¹². In the human body, HA is synthesized by three Hyaluronan synthase isoenzymes (HAS1, HAS2, and HAS3) ¹³. These isoenzymes differ in kinetic characteristics, the half-life of activity, and product size ¹⁴. HAS1 is the least active

isoenzyme with the lowest rate of synthesis of HA, and quickly loses its activity¹¹. HAS2 is the main hyaluronan synthetic enzyme for human survival, which synthesizes long HA chains. HAS1 and HAS2 produce large-sized hyaluronic acid. HAS3 is the most active HAS isoenzyme synthesizing HA with low molecular weight in the specific conditions¹¹. It is widely known that the size of this biopolymer is the most important factor for biological activity⁸.

Thus far, HA has been extracted from animal waste, including bovine eyes and rooster combs¹⁵⁻¹⁷. Currently, this procedure is being replaced by production through bacterial fermentation¹⁸⁻²². These mentioned methods suffer from some serious drawbacks, including possible contamination with the animal or bacterial toxins, expensive investment in scaling-up production systems, and the time-consuming processes of extraction and purification. Several attempts have been made to synthesize endotoxin-free HA by recombinant hosts such as *Lactococcus*²³, *Bacillus subtilis*^{24,25}, *Escherichia coli*²⁶ and *Corynebacterium glutamicum*²⁷. However, up to now, there has been no heterologous bacterial host producing as much HA as the natural ones, and the limitations of bacterial systems still exist.

Hence, there is a growing attempt to find an excellent bioreactor for HA production. In recent years, eukaryotic organisms, including yeasts such as *Saccharomyces cerevisiae*²⁸ and *Pichia pastoris*²⁹, and plant cell cultures have been studied³⁰.

Plants have become more useful for the production of Biopharmaceuticals compared to microbial sources³¹⁻³³. Using plant expression systems offers some advantages. For example, low cost, no risk of endotoxins and human pathogens, high stability of engineered product and simple and quick plant scale-up capacity^{34,35}. Furthermore it is much easier to produce, extract, and purify from plants than animal or bacterial sources^{36,37}. HA precursors (UDP-glucuronic acid and UDP-N-Acetylglucosamine) are produced in plants, along the glycolysis and pentose phosphate pathways (Fig. 1)³⁸. Nevertheless, no enzyme with hyaluronan synthase activity is found in plants³⁹.

A. rhizogenes-mediated transformation, can introduce the desired gene into the plant after infection and induce hairy roots from the wounded surface of explants^{40,41}. Hairy root systems have been used as attractive platforms for the production of biomedical products known as molecular farming^{31-33,42}. The most significant advantage of hairy roots is that they often have a higher production capacity than other plant systems⁴³.

This study revealed that, the human *HAS2* was successfully expressed in *Nicotiana tabacum* hairy roots. Conclusively elucidated that recombinant hHAS2 has a tremendous potential activity to synthesize a high molecular weight HA (HMW-HA) in tobacco hairy roots, which was determined by GPC analysis. Besides, a simple method for the extraction of HA from plants was developed.

Results

***A. rhizogenes*-mediated transformation**

The construct pBI121-hHAS2 was transformed into *A. rhizogenes* (strain ATCC AR15834) by electroporation. The presence of the construct harboring *HAS2* in transformed *A. rhizogenes* was verified by PCR amplification of a 203-bp fragment related to *HAS2* using specific primers (Fig. 2A). For further analysis, the plasmid pBI121-HAS2 extracted from transformed bacteria was digested using *Bam*HI and *Sac*I enzymes and *Kpn*I and *Hind*III enzymes that released 1666-bp and 2421-bp fragments respectively (Fig. 2B and 2C).

Molecular analysis of hairy roots

About 10 days after the transformation of tobacco explants with recombinant *A. rhizogenes* containing the constructs pBI121-hHAS2, the roots began to develop and their growth continued in the selected medium (Fig. 3A). Roots were sub-cultured every 14 days by transferring one piece of root to the new selection medium. The purpose was the regeneration of transgenic hairy root clones and the elimination of agrobacterium contamination, respectively. Then each transgenic hairy root line was transferred to the Erlenmeyer for further growth (Fig. 3B).

The putative transformants grown in the selection medium were first examined for the absence of *A. rhizogenes* contamination, by PCR using primers specific for *virG*, a bacterial gene that does not integrate into plant genomes. No specific fragment was amplified from these roots whereas, in *A. rhizogenes* as a positive control, the expected *virG* fragment was detected by PCR amplification of a 529-bp fragment (Fig. 4A). Hairy roots were rendered bacteria-free by transferring them weekly to fresh medium.

The presence of the *rolB* in the genome of hairy roots was confirmed by PCR amplification of a 194-bp fragment using specific primers (Fig. 4B). The *rolB* gene showed to be very efficient in promoting root formation.

The 203-bp fragment belonging to *HAS2* was amplified using DNA and cDNA templates extracted from the tobacco hairy roots using specific primer pairs. The presence of *HAS2* was confirmed in selected lines (Fig. 4C).

Through the PCR, as performed by specific primers, it was confirmed that all hairy root lines had been genetically transformed, and there was no sign of bacterial contamination in the hairy root lines.

Assessment of rhHAS2 in transgenic lines

The *in vitro* quantitative measurement of hHAS2 in transgenic lines was performed using a human HAS2 ELISA kit. This kit is based on the biotin double antibody sandwich technology with high sensitivity and excellent specificity for detection of hHAS2. According to the instruction of this kit, no significant cross-reactivity or interference between hHAS2 and other analogs was detected. The results confirmed the presence of hHAS2 in all transgenic hairy roots. The highest concentrations of hHAS2 produced in transgenic hairy root lines was measured at 65.72 ng/kg (wet weight).

Assessment of HA in transgenic lines

Among all of the obtained transgenic hairy root lines, 5 lines with highest hHAS2 concentration were selected to evaluate for HA content using HA ELISA kit, CTM (cetyltrimethylammonium bromide turbidimetric), and Carbazole methods. For more accuracy, each sample was replicated 3 times and the average was considered the final result. The HA was verified and quantified using a human HA ELISA kit. The results showed the maximum concentration of 0.56 g/kg (wet weight) As shown in (Table 1), the Carbazole and CTM assays resulted about two times higher than the HA ELISA kit.

Hairy roots no.	Carbazole	CTM	HA ELISA
1	0.83	0.77	0.41
2	1.3	1.12	0.56
3	0.7	0.6	0.31
4	0.78	0.72	0.39
5	0.72	0.63	0.37

Table 1. Comparison of the concentration hyaluronic acid measured by Carbazole, CTM, and HA ELISA Methods. Quantities are in g/kg (wet weight).

HA molecular weight determination

To determine the molecular weight of extracted hyaluronic acid, aqueous GPC was performed. Standard polysaccharide (containing ~0.8 MDa) utilized for comparison was represented. The first pink peak eluted in retention 6.5 min, before the first standard pick, showed HMW-HA, approximately > 0.8 Da. The short peak was due to the low concentration of HA in the total volume of the solution. Respectively the second pink peak represented solvent peak matched with standard solvent peak (Fig. 5). The GPC analysis of HA produced by transgenic hairy roots showed HMW-HA > 0.8 MDa.

Surface characteristics of HA scaffolds

The morphological three-dimensional features of HA scaffolds were investigated using a scanning electron microscope (SEM). High magnification imaging of HA scaffolds revealed the huge number of intertwined fibers as a sponge with many pits and grooves on its structure. These fibers were similar to fungus hyphae. The same observation was reported by Hamad, 2017 (Fig. 6) ⁴⁴.

DPPH radical scavenging activity

DPPH (1, 1-diphenyl-2-picryl-hydrazyl) is a stable free radical that significantly its absorbance decreases when exposed to the radical scavengers. The antioxidant potential of HA produced by transgenic hairy roots was measured regarding their efficiency in scavenging free radicals generated by DPPH. Results revealed that the isolated HA with a concentration of 0.56 g/kg (wet weight) exhibited an acceptable level of antioxidant activity of 46%, which implies its possible application in pharmaceutical products ^{45,46}.

Discussion

Many studies have shown that the high-level production of recombinant products depends on the elements of the gene cassette, such as a strong promoter and appropriate polyadenylation site, which are often derived from the 35S gene of cauliflower mosaic virus (CaMV) ⁴¹. In the present study, the rhHAS2 expressed in transgenic hairy roots under the control of the strong CaMV 35S promoter produced a significant amount of the HMW-HA. The second important factor is codon-usage compatibility between the target-gene sequence and the genome of the expression host ⁴⁷. The lack of codon optimization can reduce the level of gene expression. This is due to that the deficit of accessible tRNAs in the host, cause stopping the elongation of the target peptide or resulting in incomplete translation ⁴¹. It is accepted that the codon optimization of CDS based on the host expression system used plays an important role in heterologous gene expression ⁴⁷. In the current study, the *hHAS2* CDS was codon-optimized for expression in *N. tabacum* hairy roots, and the results demonstrated that the rhHAS2 was efficiently expressed in all of the transgenic hairy roots. The third and the most important factor altering the yield of recombinant proteins is subcellular targeting, which changes the folding, assembling, and PTM processes ⁴⁷. The KDEL tetrapeptide ER-retention signal was fused to the C-terminus of hHAS2 to target the recombinant protein to the ER lumen, which is a subcellular location that is protected from the host protease and contains a chaperone and glycosylation system for right folding and stability. Furthermore, protein glycosylation occurs only in the endomembrane system and this modification is necessary for the correct functioning of many proteins of human origin ⁴⁷.

In recent years, eukaryotic organisms, including yeasts such as *Saccharomyces cerevisiae* ²⁸, *Pichia pastoris* ²⁹, and plant cell cultures transformed with a chloroviral hyaluronan synthase (cvHAS) gene have been studied (Table 2) ³⁰. Plant cell cultures usually grow slower than [bacterial or yeast cells](#) and normally have lower recombinant [protein expression](#) levels. Furthermore, due to [somaclonal variation](#) callus cells in some conditions suffer from genetic instabilities ⁴⁸.

Among known human HASs, HAS2 is particularly responsible for the synthesis of HMW-HA in humans ⁴⁹. This study is the first to report **the human HAS2** gene was successfully introduced to the plant expression system. The rhHAS2 showed an acceptable activity in order to high molecular weight HA production > 0.8 Mda.

Our results confirmed the high transformation efficiency of *A. rhizogenes*, with the rapid scaling-up of transgenic hairy root production. Hairy root expression systems offer some advantages over other systems such as, low production costs, easy culture, biological safety, high growth rate, genetic stability, and growth in defined media that are simple and free of hormones. Because of the ability of hairy roots to grow in the absence of plant-growth regulators, the possibility of hormone-induced chromosomal abnormalities was eliminated ⁶³. Then the phenotype and molecular appearances of the hairy root lines were preserved over a long time. Similar to suspended cells, hairy roots can be cultured in a controlled environment that is appropriate for the production of high-value HA⁶³. Several factors such

as a genotype, wounding of the plant tissue and, synthesis of phenolic *vir* gene inducers by the plant can limit the transformation of the hairy roots ^{50,51}. The transgenic hairy root clones showed slight phenotypic differences from non-transformed roots, which is an important parameter for scaling ⁵².

The HA extracted from transgenic hairy roots was verified and quantified using a human HA ELISA kit. The results showed that the maximum HA concentration level of 0.56 g/kg (wet weight). The HA ELISA kit is a quantitative enzyme-linked immunoassay designed for the *in vitro* measurement of HA levels. Its specificity is based on the use of proteins or proteoglycans that detect and bind to the HA but no other biological molecules. In previous studies, non-specific quantification methods such as CTM and Carbazole have often been used to determine HA in laboratory settings (Table 2). Therefore, to compare the hyaluronic acid content in this study with previous studies in this field, other methods such as CTM and Carbazole were also examined (Table 1).

Organism	SourceHAS	HA Concentration (g/l)	Molecular Weight (MDa)	Quantification Method	Ref.
<i>Enterococcus faecalis</i>	<i>S. pyogenes</i>	0.69	***	Carbazole	53
<i>Escherichia coli</i>	<i>S. pyogenes</i>	0.08	***	Carbazole	53
<i>Escherichia coli</i>	Human	***	***	***	54
<i>Bacillus subtilis</i>	<i>S. equisimilis</i>	0.81	1.2	Carbazole	55
<i>Lactococcus lactis</i>	<i>S. zooepidemicus</i>	0.65	***	HPLC	26
<i>Bacillus subtilis</i>	<i>S. zooepidemicus</i>	0.84	***	Carbazole	25
<i>Agrobacterium sp.</i>	<i>P. multocida</i>	0.3	0.7-2	Carbazole	56
<i>Escherichia coli</i>	<i>P. multocida</i>	0.55	1.5	Carbazole	57
<i>Lactococcus lactis</i>	<i>S. zooepidemicus</i>	0.59	0.88	Radioimmunoassay	58
<i>Lactococcus lactis</i>	<i>S. zooepidemicus</i>	0.11	2.8	Carbazole	59
<i>Tobacco Cells</i>	<i>Chlorovirus</i>	***	***	***	30
<i>Bacillus subtilis</i>	<i>P. multocida</i>	***	5.43	Carbazole	60
<i>Lactococcus lactis</i>	<i>S. zooepidemicus</i>	0.14	4.3	Carbazole	61
<i>Pichia pastoris</i>	<i>Xenopus laevis</i>	0.2	0.25	Carbazole	29
<i>Streptomyces albulus</i>	<i>S. zooepidemicus</i>	5.1	2	Carbazole	62
<i>Bacillus subtilis</i>	<i>S. zooepidemicus</i>	2.05	1.76	Carbazole	63
<i>Corynebacterium glutamicum</i>	<i>S. equisimilis</i>	0.14	***	CTM	27
<i>Corynebacterium glutamicum</i>	<i>S. equisimilis</i>	5.4	1.28	CTM	64
<i>Bacillus subtilis</i>	<i>S. equisimilis</i>	0.48	1.95	Carbazole	65
<i>Kluyveromyces lactis</i>	<i>P. multocida</i>	1.89	2.09	Carbazole	66
<i>Tobacco hairy roots</i>	Human	0.56	>0.8	ELISA KIT	This study

Table 2. Engineered organisms for HA production.*** Information was not available.

As shown in (Table 1), The results obtained from Carbazole and CTM methods were twice as high as ELISA method. This is due to the low specificity of CTM and Carbazole methods and the possibility of reacting with other polysaccharides in the tissue ⁶⁷. The CTM and Carbazole methods are accurate but have many drawbacks. These methods are influenced by residual concentrations of salts and carbon sources present in the media cultures that are co-purified along with HA. The CTM assay even showed that it is more accurate than the Carbazole assay ⁶⁸.

HA turnover depends on the *activity* of hyaluronidase *enzyme*. Hyaluronidase specifically hydrolyzes the β -1,4 linkages of the HA molecule ⁶⁹. Due to the lack of hyaluronidase, the engineered plants are a reliable alternative for HA production.

Further studies are necessary to accelerate the HA production, in this context over expression of upstream genes that catalyze precursor preparation reactions could be effective.

Conclusions

According to the present study, a rapid and efficient plant regeneration system has been developed to produce hyaluronic acid, which is not naturally produced in plants. Transgenic tobacco hairy roots provide a promising and safe system for HA production. Besides, HA accumulated in tubers, fruits, and roots is easily collected and can be used as food or for use in many cosmetic products, which in many cases reduces the cost of extraction and purification. Since HA production in transgenic plants can be provided at a low cost, current research is expected to expand rapidly in the industry.

Materials And Methods

Construction of the pBI121-hHAS2 expression vector

The nucleotide sequences of *HAS2* encoding hyaluronic acid synthase 2 (accession number: [NM_005328.3](#)) were obtained from the NCBI database. To designing of the gene construct, the Kozak sequence (acaaaatggc) and endoplasmic reticulum retention signal peptide KDEL (Lys-Asp-Glu-Leu) were added to the 5' and 3' end of the coding region of *HAS2* respectively ^{32,52}. Besides, *Bam*HI (5'site) and *Sac*I (3'site) restriction sites were added for directional cloning into the same sites of the plant expression binary vector pBI121. Then, RNA destabilizing motifs and repeated sequences were dropped. Finally, the designed sequences were optimized based on the *N. tabacum* codon usage table using Gene Designer 2.0. The gene construct was chemically synthesized (General Biosystems, USA) and cloned into pUC57. Then pUC57-hHAS2 recombinant construct was digested by the *Bam*HI and *Sac*I. Then the hHAS2 was cloned in the pBI121 vector and a recombinant pBI121-hHAS2 expression vector was designed. In this vector, which contains EPSPS gene conferring resistance to glyphosate (5-enolpyruvylshikimate-3-phosphate), the expression of the hHAS2 CDS was under the control of the CaMV 35S promoter and the nopaline synthase (NOS) terminator (Fig 7) ⁷⁰.

Transformation of expression vectors into *E. coli* and *A. rhizogenes*

The pBI121-hHAS2 expression vector was diluted 10 times in sterile distilled water to a final concentration of 5 μ g/ μ L. Consequently, 2 μ L of this sample was employed to transform *E. coli* strain DH5 α using the electroporation method. These bacteria were then spread on Luria-Bertani (LB) agar (5 g of yeast extract, 10 g of tryptone, 1.5% agar, and 5 g of sodium chloride in 1 L of distilled water, pH 7) supplemented with 50 μ L of 50 mg/mL kanamycin added to 50 mL of LB solid medium ⁷¹. The plates

were incubated at 37 °C overnight. Single colonies were selected and cultured in liquid LB medium supplemented with 50 mg/L kanamycin. The cultures were incubated at 37 °C overnight. The transformation of the colonies was confirmed using a specific PCR. The plasmid was extracted from transformed *E. coli* using the GF-1 plasmid DNA extraction kit (Vivantis, Malaysia). Then 2 µL of the recombinant plasmid (at the concentration of 5 µg/µL) were transformed into electrocompetent cells (100 µL) of *A. rhizogenes* bearing the desired binary vector and the appropriate antibiotics (OD 600 nm = 0.8) using the electroporation method. The cells were incubated at 28 °C in the dark for 2 h. Then the transformed *A. rhizogenes* bacteria were spread on LB solid medium supplemented with 50 mg/L kanamycin and 100 mg/L rifampicin. The cells were incubated at 28 °C in the dark for 48 h. The verification of *A. rhizogenes* colonies was performed by specific PCR and by digestion of the extracted plasmid using *Bam*HI, *Sac*I, *Kpn*I, and *Hind*III specific enzymes.

Plant materials

Seeds of *N. tabacum* L. cv. Turkish were obtained from the Plant Virology Research Center, College of Agriculture, Shiraz University, Shiraz, Iran. The seeds were sterilized by being immersed in 70% (v/v) ethanol for 30 sec. Afterward, they were soaked in 2% hypochlorite sodium for 10 min. Next, they were rinsed five times using sterile distilled water. Finally, the seeds were cultured in hormone-free MS medium solidified with 7 g/L plant agar with a pH 5.8 and autoclaved at 121°C for 20 min⁷². The cultures were maintained at 25 °C for 2 weeks with a 16/8 h light/dark photoperiod until true leaves were just emerged and used as a source of explants for hairy roots induction.

Induction and establishment of hairy roots

The aseptic leaf explants about 0.5-1 cm long were cut with a sterile scalpel and soaked in both transformed *A. rhizogenes* and non-transformed *A. rhizogenes* (as negative control) cultures with an OD_{600 nm} of 0.5 for 1 minute. To remove the excess bacteria, the soaked explants were placed on sterile blotting paper. Next, the explants were cultured on an optimized co-cultivation medium (MS medium containing 100 µM acetosyringone solidified with 0.8% agar) under dark conditions at 25 °C⁷³. After two days, explants were transferred and placed upside down onto MS medium supplemented with 30 mg/L meropenem and 0.5 mg/L glyphosates in the dark at 25 °C for 2 weeks⁴¹. Each hairy root developed at the marginal edges of the leaf fragments was maintained as a single line. Hairy roots were made bacteria-free by transferring to a fresh medium every two weeks, the hairy root lines were chopped into 3-4 cm pieces and sub-cultured on MS solid medium at the concentrations noted above and were incubated at 25°C in the dark.

Molecular analysis of hairy root lines

Total genomic DNA was extracted from transgenic and non-transgenic (negative control) hairy roots using a modified CTAB method⁷⁴. Isolated DNA was used in PCR analysis for screening hairy roots. This

was done by specific primers for *HAS2* (oligonucleotide primers were designed using Allele ID 7 and Vector NTI 11 software and synthesized by MWG Biotech (Germany) (Table 3).

The PCR mixture (20 µl) contained 7 µL of the master mix, 1 µL of each *HAS2* specific primers (10 pmol), 10 µL of H₂O, and 1 µL of genomic DNA (100 ng).

PCR for *HAS2* was carried out by amplifying with an initial denaturation at 94°C for 5 min followed by 30 cycles of amplification with each cycle consisting of the following steps: 94 °C for 1 min, 58.7 °C for 30 s, and 72 °C for 45 s with a final extension of 72°C for 10 min. The PCR products were analyzed by electrophoresis on 1% agarose gel in 0.5% TBE buffer.

To detect the transgenic nature of the hairy roots, PCR was performed using a set of *rolB* specific primers (Table 3) to amplify the 194 bp fragment. The PCR mixture and conditions were as described earlier. The PCR product was analyzed by 10 g/L agarose gel electrophoresis.

Furthermore, primers that are specifically designed for the amplification of *virG* (Table 3) were used for confirming the elimination of infection by *A. rhizogenes*.

A 20 µl of PCR mixture contained 1 µl of each *virG* specific primers (10 pmol), 7 µL of the master mix, 10 µL of H₂O, and 1 µL of genomic DNA (100 ng). The PCR conditions were 94°C for 5 min followed by 30 cycles of amplification with each cycle consisting of the following steps: 94°C for 30 s, 50.4°C for 20 s, and 72°C for 40 s with a final extension of 72°C for 10 min. The PCR product was analyzed by 10 g/L agarose gel electrophoresis.

Total RNA was extracted from transgenic hairy roots and non-transgenic ones using a Dena-zist RNA isolation kit (Tehran, Iran) according to the manufacturer's instructions. The concentration of the RNA was measured using a Nanodrop device (Thermo Fisher Scientific, USA). The integrity and quality of the RNA were evaluated by visual observation of the 28S and 18S rRNA bands on a 1% agarose gel. Then the extracted RNA was treated with RNase-free DNase (Thermofisher, USA). Afterward, 1 µg of DNase-treated RNA of each sample was used to synthesize the first strand of cDNA using oligo-dT primers (Thermofisher, USA). According to the manufacturer's instructions. The cDNA samples were stored at -20 °C until use. The expression of *HAS2* was confirmed by *HAS2* specific primers using cDNA of transgenic samples as a template.

Primers	Sequence	Product length (bp)	Ta (C°)
-F <i>RolB</i>	AAGTGCTGAAGGAACAATC	194	54
-R <i>RolB</i>	CAAGTGAATGAACAAGGAAC		
-F <i>VirG</i>	CCTTGGGCGTCGTCATAC	529	55
-R <i>VirG</i>	TCGTCCTCGGTCGTTTCC		
2-F <i>HAS</i>	AACATCTGGGCGAAAAACC	203	58.7
R <i>HAS2</i> -	CGACATAAAGCGTAAGGAGCA		

Table 3. Sequences of the primers used for PCR-based characterization of transgenic hairy roots and the resulting product sizes. The primers were designed using Vector NTI 11 and Allele ID 7 software. Ta, temperature annealing; F, Forward; R, Reverse.

The transgenic hairy roots were transferred to a 250 ml Erlenmeyer flask containing 30 ml of hormone-free MS liquid medium on an orbital shaker at 90 rpm in the dark at 28 ± 1 °C, for one month. The medium was refreshed weekly.

The hHAS2 detection and quantification

The *in vitro* quantitative measurement of the hHAS2 enzyme was executed using the hHAS2 ELISA kit according to the manufacturer's instructions (cat.No. E1578Hu/China). For this purpose, transgenic hairy roots were grounded using liquid nitrogen, then the powder (1 g wet weight) was suspended in (1:1) 0.1 M NaNO₃ (w/v) and vortexed vigorously. Next, the cellular extracts were centrifuged at 7,000 rpm for 20 min at 4 °C. Afterward, the supernatant was collected. According to the concentration of standards and the amount of optical density (OD), the linear regression equation of the standard curve was calculated. The concentration of HAS2 in the samples was then determined by comparing the OD of the samples to the standard curve.

Extraction and pre-purification of HA

A number of transgenic hairy roots in which the presence of hHAS2 was confirmed were used to extract HA. Hairy root tissues were grounded using liquid nitrogen, and the powder (1 g wet weight) was suspended in (1:1) 0.1 M NaNO₃ (w/v) and vortexed vigorously for 2 min. Then the suspensions were centrifuged at 7,000 rpm for 20 min at 4 °C, and the supernatants were collected. After discarding cells, 1.5 volumes of ethanol were added to one volume of supernatant and the solution was kept at 4 °C for 1 hour to enhance HA precipitation. The suspensions were centrifuged at 7,000 rpm for 10 min at 4 °C and the supernatant was discarded (Fig. 8). Then the precipitate was re-dissolved in 1mL 0.1 M NaNO₃ and kept at -20 °C for further studies.

Hyaluronan detection and quantification

Quantification of HA by Carbazole method

Carbazole-based quantification involves two steps: 1) hydrolyzing HA into glucuronic acid and glycosamine with 25 mM sodium tetraborate in sulfuric acid, and 2) coloring glucuronic acid with Carbazole. First, 200 µL of diluted samples (1:10) were introduced into glass tubes filled with 1 mL of 25 mM Na₂B₄O₇ in H₂SO₄. The tubes were incubated at 100 °C for 15 min. After cooling on ice for 2 min, 40 µL of 0.125% (w/v) Carbazole in absolute ethanol was introduced to each tube and incubated at 100 °C for 15 min. After cooling, the absorbance was read at 550 nm⁶⁷. A standard curve of HA concentrations ranging from 0.1 to 0.8 g/L was plotted using 99% HA (Sigma-Aldrich, St. Louis, MO, USA). The HA concentration was calculated based on the standard calibration curve and the dilution ratio.

Quantification of HA by CTM assay

To determine the HA concentration by CTM, 200 μ L of HA samples diluted 1:10 were introduced into 1.5 tubes filled with 200 μ L of 0.1 M phosphate buffer pH 7. The tubes were incubated at 37 °C for 15 min. Then, 400 μ L of CTM reagent (2.5 g CTAB dissolved in 100 mL of 2% (w/v) NaOH at 37 °C) was added to each tube and incubated for 10 min at 37 °C. Each tube was shaken for 10 s at the beginning and the end of this incubation. Absorbance was read at 600 nm against the blank (0.1 M phosphate buffer pH 7). The slope of the standard curve was obtained using linear regression in the HA concentration range between 0.1 and 0.6 g/L using 99% HA. The HA concentration was calculated based on the standard calibration curve and the dilution ratio ⁶⁷.

Quantification of HA by ELISA kit

The amount of extracted hyaluronic acid from the transgenic hairy root lines was determined using the human HA ELISA kit (Cat.No. MBS262948 /USA) according to the manufacturer's instructions. The color depth and the content of HA in samples are positively correlated. Based on the concentration of the standards and OD value, the linear regression equation of the standard curve was calculated. The HA concentration was assayed based on the standard calibration curve and was multiplied by the dilution coefficient when calculating the results.

Determination of HA molecular weight

The molar mass of HA at a concentration of 0.56 g/kg (wet weight) was determined by GPC analysis. As a solvent, a 0.1 M NaNO₃ solution at the flow rate of 1 mL/min was used in a column system, ultra-hydrogel linear (Shimadzu LC-20A). The injection volume was 20-50 μ l with refractive index detectors and polysaccharide contained 853000 Dalton as a standard in GPC analysis was performed.

Scanning electron microscopy

SEM analysis was carried out by using a scanning electron microscope (TESCAN-Vega 3, TESCAN, Czech Republic). The extracted hyaluronic acid was poured on a coverslip and air-dried. The sample was fixed overnight in 1 mL of 3% (v/v) glutaraldehyde in phosphate buffer at 4 °C and followed by dehydrated once in 50%, 75%, 90%, and twice in 100% ethanol series for 15 min for each step, respectively. After air-drying, the coverslip was coated with a sputtered gold coating and examined under a microscope.

Antioxidant activity

The DPPH radical scavenging effect of HA was measured using DPPH (Sigma-Aldrich Brazil Ltda) ⁷⁵. Briefly, 100 μ L of 0.16 mM DPPH solution (in methanol) was added into 0.5 tubes filled with 100 μ L of HA 0.56 g/kg (wet weight). The mixture was shaken for 1min then introduced into a 96-well microplate incubated at room temperature for 30 minutes in the dark. The absorbance was measured at 517 nm using a UV-visible spectrophotometer ^{44,75}. Inhibition percentage was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{\text{A of control} - \text{A of sample}}{\text{A of control}} \times 100$$

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Figures

Figure 1

Schematic overview of the hyaluronan synthesis pathway in a transgenic plant. HA precursors are produced in plants, along the glycolysis and pentose phosphate pathway. HAS gene must be introduced into the plant to produce heterologous HA.

Figure 2

The presence of the construct harboring HAS2 in transformed *A. rhizogenes* was confirmed by PCR amplification of a 203-bp fragment and also digestion of transgenic recombinant plasmids using specific enzymes. (A) Lane M, Ruler 100bp DNA ladder Mix (Thermofisher, USA); lane 1, positive control (203-bp fragment from transformed *E. coli*); lane 2-8, transformed *A. rhizogenes* showed the amplification of 203-bp fragment; lane 9, non-transformed *A. rhizogenes* and lane 10, PCR negative control (water). (B) Lane1, digestion of recombinant *A. rhizogenes* plasmids with BamHI and SacI enzymes that released a 1666-bp fragment. (C) Digestion of recombinant *A. rhizogenes* plasmids by KpnI and HindIII enzymes that released a 2421-bp fragment.

Figure 3

Formation and elongation of *N. tabacum* hairy roots at different periods after *A. rhizogenes* infection. (A) Induction and propagation of hairy roots at the marginal edges of leaf explants grown on solid MS medium supplemented with 30 mg/L meropenem and 0.5 mg/L glyphosate at 25 °C under dark

conditions after three weeks of post-infection. (B) Growth of hairy roots cultivated in a 250 mL Erlenmeyer flask containing MS liquid medium without antibiotics for one month at 28 °C in the dark with gentle shaking.

Figure 4

The absence of *A. rhizogenes* contamination confirmed using PCR specific for *virG*. PCR amplification of a 194-bp fragment of *rolB* and 203-bp fragment of *HAS2* using DNA and cDNA derived from hairy roots. (A) Lane M: 100bp DNA ladder Mix (Thermofisher, USA), lanes 1-3, no fragment was amplified using DNA template extracted from transgenic hairy root lines; lane 4, wild-type hairy root; lane 5, water as negative control; lane 6, PCR amplification of the 529-bp fragment of *virG* using *A. rhizogenes* plasmid as a positive control. (B) lane 1, positive control (recombinant *A. rhizogenes* plasmid); lanes 2-4, DNA template extracted from transgenic hairy root lines showing the amplification of a 194-bp fragment of *rolB*; lane 5, negative control (wild-type hairy root); lane 6, water as the negative control. (C) lane 1 positive control (recombinant *A. rhizogenes* plasmid), lanes 2-4: cDNA template derived from transgenic hairy root lines showing the amplification of a 203-bp fragment of *HAS2*; lane 5, water as a negative control; lane 6, cDNA template derived from wild-type hairy root.

Figure 5

analysis of the extracted HA. The first pink peak showed HMW-HA < 853000 Da.

Figure 6

Scanning electron microscopy (SEM) of hyaluronic acid scaffolds at low (A) and high (B,C,D,E and F) magnification starting from 2-400 KX. The HA appeared in a scanning electron microscope as a sponge with a huge number of fibers similar to fungus hyphae at very high magnification.

Figure 7

The schematic map of the pBI121-hHAS2 expression vector. pBI121 expression vector contained the codon-optimized HAS2 gene under the control of the CaMV 35S promoter and a NOS terminator; EPSPS resistance gene; LB, T-DNA left border; RB, T-DNA right border; Restriction sites.

Figure 8

Collecting the HA using ethanol precipitation. The precipitated HA was dissolved in 0.1M NaNo₃.

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