(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 April 2007 (12.04.2007)

PCT

(10) International Publication Number
WO 2007/039314 A2

(51) International Patent Classification:
C12N 15/82 (2006.01)

(21) International Application Number:
PCT/EP2006/009773

(22) International Filing Date: 5 October 2006 (05.10.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
05090279.0 5 October 2005 (05.10.2005) EP
60/725,388 11 October 2005 (11.10.2005) US

(71) Applicant *(for all designated States except US)*: **BAYER CROPSCIENCE GMBH** [DE/DE]; Brüningstrasse 50, 65929 Frankfurt (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **FROHBERG, Claus** [DE/DE]; Pilzwald 17, 14532 Kleinmachnow (DE). **ESSIGMANN, Bernd** [DE/DE]; Menzelstrasse 26, 12157 Berlin (DE).

(74) Agent: **KOSSMANN, Jochen**; Bayer Bioscience GmbH, Hermannswerder 20a, 14473 Postsdam (DE).

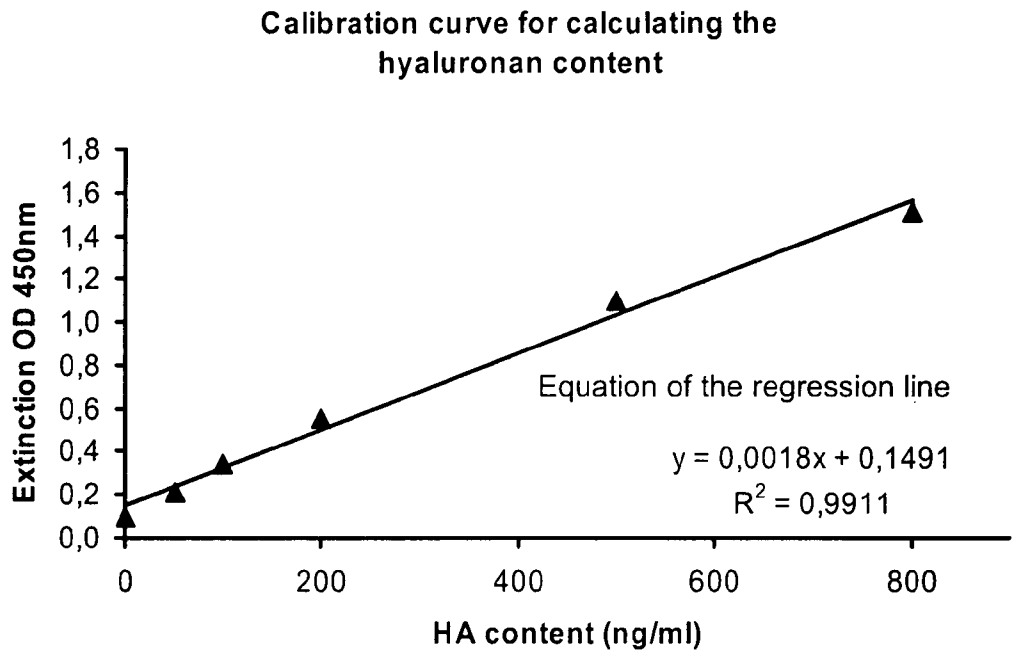
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

[Continued on next page]

(54) Title: PLANTS WITH INCREASED HYALURONAN PRODUCTION



(57) Abstract: The present invention relates to plant cells and plants which synthesize an increased amount of hyaluronan, and to methods for preparing such plants, and also to methods for preparing hyaluronan with the aid of these plant cells or plants. Here, plant cells or genetically modified plants according to the invention have hyaluronan synthase activity and additionally an increased glutamine:fructose 6-phosphate amidotransferase (GFAT) activity compared to wild-type plant cells or wild-type plants. The present invention furthermore relates to the use of plants having increased hyaluronan synthesis for preparing hyaluronan and food or feed-stuff containing hyaluronan.

WO 2007/039314 A2

Published:

— *without international search report and to be republished upon receipt of that report*

— *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Plants with increased hyaluronan production

The present invention relates to plant cells and plants which synthesize an increased amount of hyaluronan, and to methods for preparing such plants, and also to methods for preparing hyaluronan with the aid of these plant cells or plants. Here, plant cells or genetically modified plants according to the invention have hyaluronan synthase activity and additionally an increased glutamine:fructose 6-phosphate amidotransferase (GFAT) activity compared to wild-type plant cells or wild-type plants. The present invention furthermore relates to the use of plants having increased hyaluronan synthesis for preparing hyaluronan and food or feedstuff containing hyaluronan.

Hyaluronan is a naturally occurring unbranched, linear mucopolysaccharide (glucosaminoglucan) which is constructed of alternating molecules of glucuronic acid and N-acetyl-glucosamine. The basic building block of hyaluronan consists of the disaccharide glucuronic acid-beta-1,3-N-acetyl-glucosamine. In hyaluronan, these repeating units are attached to one another via beta-1,4 linkages.
In pharmacy, use is frequently made of the term hyaluronic acid. Since hyaluronan is in most cases present as a polyanion and not as the free acid, hereinbelow, the term hyaluronan is preferably used, but each term is to be understood as embracing both molecular forms.

Hyaluronan has unusual physical chemical properties, such as, for example, properties of polyelectrolytes, viscoelastic properties, a high capacity to bind water, properties of gel formation, which, in addition to further properties of hyaluronan, are described in a review article by Lapcik et al. (1998, Chemical Reviews 98(8), 2663-2684).

Hyaluronan is a component of extracellular connective tissue and bodily fluids of vertebrates. In humans, hyaluronic acid is synthesized by the cell membrane of all body cells, especially mesenchymal cells, and ubiquitously present in the body with a particularly high concentration in the connective tissues, the extracellular matrix, the

umbilical cord, the joint fluid, the cartilaginous tissue, the skin and the vitreous body of the eye (Bernhard Gebauer, 1998, Inaugural-Dissertation, Virchow-Klinikum Medizinische Fakultät Charité der Humboldt Universität zu Berlin; Fraser et al., 1997, Journal of Internal Medicine 242, 27-33).

Recently, hyaluronan was also found in animal non-vertebrate organisms (molluscs) (Volpi and Maccari, 2003, Biochimie 85, 619-625).

Furthermore, some pathogenic gram-positive bacteria (*Streptococcus* group A and C) and gram-negative bacteria (*Pasteurella*) synthesize hyaluronan as exopolysaccharides which protect these bacteria against attack by the immune system of their host, since hyaluronan is a non-immunogenic substance.

Viruses which infect single-cell green algae of the genus *Chlorella*, some of which are present as endosymbionts in *Paramecium* species, bestow upon the single-cell green algae the ability to synthesize hyaluronan after infection by the virus (Graves et al., 1999, Virology 257, 15-23). However, the ability to synthesize hyaluronan is not a feature which characterizes the algae in question. The ability of the algae to synthesize hyaluronan is mediated by an infection with a virus whose genome has a sequence coding for hyaluronan synthase (DeAngelis, 1997, Science 278, 1800-1803). Furthermore, the virus genome contains sequences coding for a glutamine: fructose 6-phosphate amidotransferase (GFAT). GFAT converts fructose 6-phosphate and glutamine into glucosamine 6-phosphate which is an important metabolite in the metabolic pathway for hyaluronan synthesis. Both genes encode active proteins which, like the hyaluronan synthase of the virus, are transcribed simultaneously in the early phase of the viral infection (DeAngelis et al., 1997, Science 278, 1800-1803, Graves et al., 1999, Virology 257, 15-23). The activity of a protein having glutamine:fructose 6-phosphate amidotransferase (GFAT) activity could be detected neither in extracts from cells not infected by a virus nor in virus-infected cells (Landstein et al., 1998, Virology 250, 388-396). Accordingly, the role of the expression of GFAT in virus-infected Chlorella cells for the hyaluronan synthesis, and whether they are required for hyaluronan synthesis, is not known.

Naturally occurring plants themselves do not have any nucleic acids in their genome which code for proteins catalyzing the synthesis of hyaluronan and, although a large number of plant carbohydrates have been described and characterized, it has hitherto not been possible to detect hyaluronan or molecules related to hyaluronan in

non-infected plants (Graves et al., 1999, Virology 257, 15-23).

The catalysis of the hyaluronan synthesis is effected by a single membrane-integrated or membrane-associated enzyme, hyaluronan synthase. The hyaluronan synthases which have hitherto been studied can be classified into two groups: hyaluronan synthases of Class I and hyaluronan synthases of Class II (DeAngelis, 1999, CMLS, Cellular and Molecular Life Sciences 56, 670-682).
The hyaluronan synthases of vertebrates are further distinguished by the identified isoenzymes. The different isoenzymes are referred to in the order of their identification using Arabic numbers (for example, hsHAS1, hsHAS2, hsHAS3).

The mechanism of the transfer of synthesized hyaluronan molecules across the cytoplasma membrane into the medium surrounding the cell has not yet been fully elucidated. Earlier hypotheses assumed that transport across the cell membrane was effected by hyaluronan synthase itself. However, more recent results indicate that the transport of hyaluronan molecules across the cytoplasma membrane takes place by energy-dependent transport via transport proteins responsible for this action. Thus, *Streptococcus* strains were generated by mutation in which the synthesis of an active transport protein was inhibited. These strains synthesized less hyaluronan than corresponding wild-type bacteria strains (Ouskova et al., 2004, Glycobiology 14(10), 931-938). In human fibroblasts, it was possible to demonstrate, with the aid of agents specifically inhibiting known transport proteins, that it is possible to reduce both the amount of hyaluronan produced and the activity of hyaluronan synthases (Prehm and Schumacher, 2004, Biochemical Pharmacology 68, 1401-1410). In which amount, if at all, transport proteins capable of transporting hyaluronan are present in plants is not known.

The unusual properties of hyaluronan offer a wealth of possibilities for application in various fields, such as, for example, pharmacy, the cosmetics industry, in the production of food and feed, in technical applications (for example as lubricants), etc. The most important applications where hyaluronan is currently being used are in the medicinal and cosmetics field (see, for example, Lapcik et al., 1998, Chemical Reviews 98(8), 2663-2684, Goa and Benfield, 1994, Drugs 47(3), 536-566).

In the medical field, hyaluronan-containing products are currently used for the intraarticular treatment of arthrosis and in ophthalmics used for eye surgery. Hyaluronan is also used for treating joint disorders in racehorses. In addition, hyaluronic acid is a component of some rhinologics which, for example in the form of eye drops and nasalia, serve to moisten dry mucous membranes. Hyaluronan-containing solutions for injection are used as analgesics and antirheumatics. Patches comprising hyaluronan or derivatized hyaluronan are employed in wound healing. As dermatics, hyaluronan-containing gel implants are used for correcting skin deformations in plastic surgery.

For pharmacological applications, preference is given to using hyaluronan having a high molecular weight.

In cosmetic medicine, hyaluronan preparations are among the most suitable skin filler materials. By injecting hyaluronan, for a limited period of time, it is possible to smooth wrinkles or to increase the volume of lips.

In cosmetic products, in particular in skin creams and lotions, hyaluronan is frequently used as moisturizer by virtue of its high water-binding capacity.

Furthermore, hyaluronan-containing preparations are sold as so-called nutraceuticals (food supplements) which can also be used in animals (for example dogs, horses) for the prophylaxis and alleviation of arthrosis.

Hyaluronan used for commercial purposes is currently isolated from animal tissues (roostercombs) or prepared fermentatively using bacterial cultures.

US 4,141,973 describes a process for isolating hyaluronan from roostercombs or alternatively from umbilical cords. In addition to hyaluronan, animal tissues (for example roostercombs, umbilical cords) also contain further mucopolysaccharides related to hyaluronan, such as chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate and heparin. Furthermore, animal organisms contain proteins (hyaladherins) which bind specifically to hyaluronan and which are required for the most different functions in the organism, such as, for example, the degradation of hyaluronan in the liver, the function of hyaluronan as lead structure for cell migration, the regulation of endocytosis, the anchoring of hyaluronan on the cell surface or the formation of hyaluronan networks (Turley, 1991, Adv Drug Delivery Rev 7, 257 ff.;

Laurent and Fraser, 1992, FASEB J. 6, 183 ff.; Stamenkovic and Aruffo, 1993, Methods Enzymol. 245, 195 ff; Knudson and Knudson, 1993, FASEB 7, 1233 ff.).

The *Streptococcus* strains used for the bacterial production of hyaluronan are exclusively pathogenic bacteria. During cultivation, too, these bacteria produce (pyrogenic) exotoxins and hemolysins (streptolysin, (in particular alpha- and beta-hemolysin) (Kilian, M.: Streptococcus and Enterococcus. In: *Medical Microbiology*. Greenwood, D.; Slack, RCA; Peutherer, J.F. (Eds.). Chapter 16. Churchill Livingstone, Edinburgh, UK: pp. 174-188, 2002, ISBN 0443070776) which are released into the culture medium. This renders purification and isolation of the hyaluronan prepared with the aid of *Streptococcus* strains more difficult. In particular for pharmaceutical application, the presence of exotoxins and hemolysins in the preparation is a problem.

US 4,801,539 describes the preparation of hyaluronan by fermentation of a mutagenized bacterial strain (*Streptococcus zooedemicus*). The mutagenized bacteria strain used no longer synthesizes beta-hemolysin. The yield achieved was 3.6 g of hyaluronan per liter of culture.

EP 0694616 describes a method for cultivating *Streptococcus zooedemicus* or *Streptococcus equi*, where, under the culture conditions employed, no streptolysin, but increased amounts of hyaluronan are synthesized. The yield achieved was 3.5 g of hyaluronan per liter of culture.

During cultivation, *Streptococcus* strains release the enzyme hyaluronidase into the culture medium, as a consequence of which, in this production system, too, the molecular weight is reduced during purification. The use of hyaluronidase-negative *Streptococcus* strains or of methods for the production of hyaluronan where the production of hyaluronidase during cultivation is inhibited are described in US 4,782,046. The yield achieved was up to 2.5 g of hyaluronan per liter of culture, and the maximum mean molecular weight achieved was 3.8×10^6 Da, at a molecular weight distribution of from 2.4×10^6 to 4.0×10^6.

US 20030175902 and WO 03 054163 describe the preparation of hyaluronan with the aid of heterologous expression of a hyaluronan synthase from *Streptococcus equisimilis* in *Bacillus subtilis*. To achieve the production of sufficient amounts of hyaluronan, in addition to heterologous expression of a hyaluronan synthase,

simultaneous expression of a UDP-glucose dehydrogenase in the *Bacillus* cells is also required. US 20030175902 and WO 03 054163 do not state the absolute amount of hyaluronan obtained in the production with the aid of *Bacillus subtilis.* The maximum mean molecular weight achieved was about 4.2×10^6. However, this mean molecular weight was only achieved for the recombinant *Bacillus* strain where a gene coding for the hyaluronan synthase gene from *Streptococcus equisimilis* and the gene coding for the UDP-glucose dehydrogenase from *Bacillus subtilis* were integrated into the *Bacillus subtilis* genome under the control of the *amy*Q promoter, where at the same time the *Bacillus subtilis*-endogenous *cxp*Y gene (which codes for a cytochrome P450 oxidase) was inactivated.

WO 05 012529 describes the preparation of transgenic tobacco plants which were transformed using nucleic acid molecules encoding for hyaluronan synthases from *Chlorella*-infecting viruses. In WO 05 012529, use was made, on the one hand, of nucleic acid sequences encoding for hyaluronan synthase of the Chlorella virus strain CVHI1 and, on the other hand, of the Chlorella virus strain CVKA1 for transforming tobacco plants. The synthesis of hyaluronan could only be demonstrated for a plant transformed with a nucleic acid encoding for a hyaluronan synthase isolated from the Chlorella virus strain CVKA1. For tobacco plants transformed with a nucleic acid sequence encoding for a hyaluronan synthase isolated from the Chlorella virus strain CVHI1, it was not possible to detect hyaluronan synthesis in the corresponding transgenic plants. The amount of hyaluronan synthesized by the only hyaluronan-producing transgenic tobacco plant in WO 05 012529 is stated as being about 4.2 µg of hyaluronan per ml of measured volume which, taking into account the description for carrying out the experiment in question, corresponds approximately to an amount of at most 12 µg of hyaluronan produced per gram of fresh weight of plant material.

Hyaluronan synthase catalyzes the synthesis of hyaluronan from the starting materials UDP-N-acetyl-glucosamine and UDP-glucuronic acid. Both starting materials mentioned are present in plant cells.

For the synthesis of UDP-N-acetylglucosamine in plant cells, WO 98 35047 describes a metabolic path where glucosamine is converted by a number of successive

enzymatically catalyzed reaction steps with formation of the metabolites N-acetyl-glucosamine, N-acetyl-glucosamine 6-phosphate, N-acetyl-glucosamine 1-phosphate into UDP-N-acetylglucosamine. An alternative metabolic path comprises a reaction of fructose 6-phosphate and glutamine giving glucosamine 6-phosphate which is subsequently converted by a number of successive enzymatically catalyzed reaction steps with formation of the metabolites glucosamine 1-phosphate and N-acetyl-glucosamine 1-phosphate into UDP-N-acetylglucosamine. The conversion of fructose 6-phosphate and glutamine into glucosamine 6-phosphate is catalyzed by a protein having glutamine:fructose 6-phosphate amidotransferase (GFAT) activity (Mayer et al., 1968, Plant Physiol. 43, 1097-1107).

WO 00 11192 describes the endosperm-specific overexpression of a nucleic acid molecule of corn encoding for a protein having the enzyme activity of a GFAT in transgenic corn plants with the aim to synthesize a cationic starch in plants which has 2-amino-anhydroglucose molecules. The metabolic path described which, according to the description of WO 00 11192 should result in 2-amino-anhydroglucose being incorporated into the starch, comprises inter alia the incorporation of UDP-glucosamine by starch synthases and/or glycogen synthases into the starch. It is stated that increased amounts of UDP-glucosamine could be detected in flour from endosperm of the transgenic corn plants in question overexpressing a nucleic acid molecule encoding for a protein having the (enzymatic) activity of a GFAT translationally fused with a plastid signal peptide. When the protein having the (enzymatic) activity of a GFAT was expressed without signal peptide, it was possible to detect an increased amount of glucosamine 1-phosphate in the corresponding flours from corn endosperm tissue. It was not possible to detect cationic starch in the transgenic plants.

The production of hyaluronan by fermentation of bacteria strains is associated with high costs, since the bacteria have to be fermented in sealed sterile containers under expensive controlled culture conditions (see, for example, US 4,897,349). Furthermore, the amount of hyaluronan which can be produced by fermentation of bacteria strains is limited by the production facilities present in each case. Here, it also has to be taken into account that fermenters, as a consequence of physical laws, cannot be built for excessively large culture volumes. Particular mention may

be made here of homogeneous mixing of the substances fed in from the outside (for example essential nutrient sources for bacteria, reagents for regulating the pH, oxygen) with the culture medium required for efficient production, which, in large fermenters, can be ensured only with great technical expenditure, if at all.

The purification of hyaluronan from animal organisms is complicated owing to the presence, in animal tissues, of other mucopolysaccharides and proteins which specifically bind to hyaluronan. In patients, the use of hyaluronan-containing medicinal preparations contaminated by animal proteins can result in unwanted immunological reactions of the body (US 4,141,973), in particular if the patient is allergic to animal proteins (for example chicken egg white). Furthermore, the amounts (yields) of hyaluronan which can be obtained from animal tissues in satisfactory quality and purity are low (croosterscomb: 0.079% w/w, EP 0144019, US 4,782,046), which necessitates the processing of large amounts of animal tissues. A further problem in the isolation of hyaluronan from animal tissues consists in effect that the molecular weight of hyaluronan during purification is reduced since animal tissues also contain a hyaluronan-degrading enzyme (hyaluronidase).

In addition to the hyaluronidases and exotoxins mentioned, *Streptococcus* strains also produce endotoxins which, when present in pharmacological products, pose risks for the health of the patient. In a scientific study, it was shown that even hyaluronan-containing medicinal products on the market contain detectable amounts of bacterial endotoxins (Dick et al., 2003, Eur J Opthalmol. 13(2), 176-184). A further disadvantage of the hyaluronan produced with the aid of *Streptococcus* strains is the fact that the isolated hyaluronan has a lower molecular weight than hyaluronan isolated from roostercombs (Lapcik et al. 1998, Chemical Reviews 98(8), 2663-2684). US 20030134393 describes the use of a *Streptococcus* strain for producing hyaluronan which synthesizes a particularly pronounced hyaluronan capsule (supercapsulated). The hyaluronan isolated after fermentation had a molecular weight of 9.1×10^6. However, the yield was only 350 mg per liter.

Some of the disadvantages of producing hyaluronan by bacterial fermentation or by isolation from animal tissues can be avoided by producing hyaluronan using

transgenic plants; however, the currently achieved amounts of hyaluronan which can be produced using transgenic plants would require a relatively large area under cultivation to produce relatively large amounts of hyaluronan. Furthermore, the isolation or purification of hyaluronan from plants having a lower hyaluronan content is considerably more complicated and costly than the isolation or purification from plants having a higher hyaluronan content.

Although hyaluronan has unusual properties, it is, owing to its scarcity and the high price, rarely, if at all, used for industrial applications.

Accordingly, it is an object of the present invention to provide means and methods which permit the provision of hyaluronan in sufficient amounts and quality and which make it possible to provide hyaluronan even for industrial applications and applications in the field of food and feed.

This object is achieved by the embodiments outlined in the claims.

Thus, the present invention relates to genetically modified plant cells or genetically modified plants having a nucleic acid molecule, stably integrated into their genome, encoding for a hyaluronan synthase, characterized in that said plant cells or said plants additionally have increased activity of a protein having an (enzymatic) glutamine:fructose 6-phosphate amidotransferase (GFAT) activity compared to corresponding not genetically modified wild-type plant cells or not genetically modified wild-type plants.

Here, the genetic modification of genetically modified plant cells according to the invention or genetically modified plants according to the invention can be any genetic modification resulting in a stable integration of a nucleic acid molecule encoding for a hyaluronan synthase into a plant cell or a plant and increasing the activity of a protein having the (enzymatic) activity of a GFAT in genetically modified plant cells or genetically modified plants, compared to corresponding not genetically modified wild-type plant cells or not genetically modified wild-type plants.

In the context of the present invention, the term "wild-type plant cell" is to be understood as meaning plant cells which served as starting material for the preparation of the genetically modified plant cells according to the invention, i.e. their genetic information, apart from the genetic modifications introduced and resulting in a stable integration of a nucleic acid molecule encoding for a hyaluronan synthase and increasing the activity of a protein having the activity of a GFAT, corresponds to that of a genetically modified plant cell according to the invention.

In the context of the present invention, the term "wild-type plant" is to be understood as meaning plants which served as starting material for the preparation of the genetically modified plants according to the invention, i.e. their genetic information, apart from the genetic modifications introduced and resulting in a stable integration of a nucleic acid molecule encoding for a hyaluronan synthase and increasing the activity of a protein having the activity of a GFAT, corresponds to that of a genetically modified plant according to the invention.

In the context of the present invention, the term "corresponding" means that, when a plurality of objects are compared, the objects in question which are compared to one another have been kept under the same conditions. In the context of the present invention, the term "corresponding" in the context of wild-type plant cells or wild-type plants means that the plant cells or plants compared to one another were cultivated under the same cultivation conditions and that they have the same (culture) age.

In the context of the present invention, the term "hyaluronan synthase" (EC 2.4.1.212) is to be understood as meaning a protein which synthesizes hyaluronan from the substrates UDP-glucuronic acid (UDP-GlcA) and N-acetyl-glucosamine (UDP-GlcNAc). The hyaluronan synthesis is catalyzed according to the reaction schemes below:

nUDP-GlcA + nUDP-GlcNAc → beta-1,4-[GlcA-beta-1,3-GlcNAc]$_n$ + 2 nUDP

Nucleic acid molecules and corresponding protein sequences coding for hyaluronan synthases have been described, inter alia, for the following organisms: rabbit

(*Oryctolagus cuniculus*) ocHas2 (EMBL AB055978.1, US 20030235893), ocHas3 (EMBL AB055979.1, US 20030235893); baboon (*Papio anubis*) paHas1 (EMBL AY463695.1); frog (*Xenopus laevis*) xlHas1 (EMBL M22249.1, US 20030235893), xlHas2 (DG42) (EMBL AF168465.1), xlHas3 (EMBL AY302252.1); human (*Homo sapiens*) hsHAS1 (EMBL D84424.1, US 20030235893), hsHAS2 (EMBL U54804.1, US 20030235893), hsHAS3 (EMBL AF232772.1, US 20030235893); mouse (*Mus musculus*), mmHas1 (EMBL D82964.1, US 20030235893), mmHAS2 (EMBL U52524.2, US 20030235893), mmHas3 (EMBL U86408.2, US 20030235893); cattle (*Bos taurus*) btHas2 (EMBL AJ004951.1, US 20030235893); chicken (*Gallus gallus*) ggHas2 (EMBL AF106940.1, US 20030235893); rat (*Rattus norvegicus*) rnHas 1 (EMBL AB097568.1, Itano et al., 2004, J. Biol. Chem. 279(18) 18679-18678), rnHas2 (EMBL AF008201.1); rnHas 3 (NCBI NM_172319.1, Itano et al., 2004, J. Biol. Chem. 279(18) 18679-18678), horse (*Equus caballus*) ecHAS2 (EMBL AY056582.1, GI:23428486), pig (*Sus scrofa*) sscHAS2 (NCBI NM_214053.1, GI:47522921), sscHas 3 (EMBLAB159675), zebra fish (*Danio rerio*) brHas1 (EMBL AY437407), brHas2 (EMBL AF190742.1) brHas3 (EMBL AF190743.1); *Pasteurella multocida* pmHas (EMBL AF036004.2); *Streptococcus pyogenes* spHas (EMBL, L20853.1, L21187.1, US 6,455,304, US 20030235893); *Streptococcus equis* seHas (EMBL AF347022.1, AY173078.1), *Streptococcus uberis* suHasA (EMBL AJ242946.2, US 20030235893), *Streptococcus equisimilis* seqHas (EMBL AF023876.1, US 20030235893); *Sulfolobus solfataricus* ssHAS (US 20030235893), *Sulfolobus tokodaii* stHas (AP000988.1), *Paramecium bursaria Chlorella* Virus 1, cvHAS (EMBL U42580.3, PB42580, US 20030235893).

In the context of the present invention, the term "glutamine:fructose 6-phosphate amidotransferase (GFAT)" (E.C. 2.6.1.16), in the expert literature also referred to as glucosamine synthase, is to be understood as meaning a protein which synthesizes, from the substrates glutamine and fructose 6-phosphate (Fruc-6-P) glucosamine 6-phosphate (GlcN-6-P). This catalysis proceeds according to the following reaction scheme:

Glutamine + Fruc-6-P → GlcN-6-P + Glutamate

In particular in animal organisms, it was possible to demonstrate two different isoforms of proteins having the (enzymatic) activity of a GFAT (referred to as GFAT-1 and GFAT-2, respectively, in the literature). Hu et al. (2004), J. Biol. Chem. 279(29), 29988-29993 describe differences of the respective proteins from the mouse: in addition to differences in the tissue-specific expression of the proteins in question having the (enzymatic) activity of a glutamine:fructose 6-phosphate amidotransferase 1 (GFAT-1) and a glutamine:fructose 6-phosphate amidotransferase 2 (GFAT-2), it was possible to show that both isoforms are regulated by phosphorylation by a cAMP-dependent protein kinase. The activity of a protein having the (enzymatic) activity of a GFAT-1 is inhibited by phosphorylation of a conserved serine residue (serine 205 in the GFAT-1 from the mouse, GenBank Acc No.: AF334736.1) of the amido acid sequence in question, whereas the activity of a protein having the activity of a GFAT-2 is increased by phosphorylation of a conserved serine residue (serine 202 in the GFAT-2 from the mouse, GenBank Acc No.: NM_013529) of the amino acid sequence in question. Both proteins having the activity of a GFAT-1 and proteins having the activity of a GFAT-2 are inhibited in a concentration-dependent manner by UDP-N-acetylglucosamine; however, for a protein having the activity of a GFAT-2, the inhibition by UDP-N-acetylglucosamine is lower (maximum reduction of activity by UDP-N-acetylglucosamine about 15%) compared to a protein having the activity of a GFAT-1 (maximum reduction of activity by UDP-N-acetylglucosamine about 51% or 80%). There are indications that the inhibition of a protein having the activity of a GFAT-1 in animal organisms is based on the fact that at elevated UDP-N-acetylglucosamine concentrations there is an O-glucose-N-acetylglucosamine glycosylation of the proteins in question. Whether a regulation of the activity of proteins by O-glycosylation also takes place in plant cells is not yet fully understood (Huber und Hardin, 2004, Current Opinion in Plant Biotechnology 7, 318-322).

In the context of the present invention: the term “glutamine:fructose 6-phosphate amidotransferase-1 (GFAT-1)” is to be understood as meaning a protein which has the activity of a GFAT and whose activity is inhibited by phosphorylation by a cAMP-dependent protein kinase.

In the context of the present invention, the term “glutamine:fructose 6-phosphate

amidotransferase-2 (GFAT-2)" is to be understood as meaning a protein which has the activity of a GFAT and which is activated by phosphorylation by a cAMP-dependent protein kinase.

In the context of the present invention, the term "glutamine:fructose 6-phosphate amidotransferase (GFAT)" is used as a comprehensive term which includes all proteins having the activity of a GFAT. Accordingly, it also comprises proteins referred to in the literature as "glutamine:fructose 6-phosphate amidotransferase-1 (GFAT-1)" or as "glutamine:fructose 6-phosphate amidotransferase-2 (GFAT-2)", but is not limited to these.

In the context of the present invention, the term "increased activity of a protein having the (enzymatic) activity of a GFAT" means an increased expression of endogenous genes coding for proteins having the activity of a GFAT and/or an increased amount of transcripts coding for proteins having the activity of a GFAT and/or an increased amount of protein having the activity of a GFAT in the cells and/or an increased enzymatic activity of proteins having the activity of a GFAT in the cells.

An increased expression can be determined, for example, by measuring the amount of transcripts coding for a protein having the activity of a GFAT, for example by Northern blot analysis or RT-PCR. Here, an increase preferably means an increase in the amount of transcripts compared to corresponding not genetically modified wild-type plant cells or not genetically modified wild-type plants by at least 50%, in particular by at least 70%, preferably by at least 85% and particularly preferably by at least 100%. An increase of the amount of transcripts coding for a protein having the activity of a GFAT also means that plants or plant cells having no detectable amounts of transcripts coding for a protein having the activity of a GFAT have, after genetic modification according to the invention, detectable amounts of transcripts coding for a protein having the activity of a GFAT.

An increase in the amount of protein having the activity of a GFAT resulting in an increased activity of these proteins in the plant cells in question can be determined, for example, by immunological methods, such as Western blot analysis, ELISA

(Enzyme Linked Immuno Sorbent Assay) or RIA (Radio Immune Assay). Methods for preparing antibodies reacting specifically with a particular protein, i.e. binding specifically to said protein, are known to the person skilled in the art (see, for example, Lottspeich and Zorbas (Eds.), 1998, Bioanalytik [Bioanalysis], Spektrum akad. Verlag, Heidelberg, Berlin, ISBN 3-8274-0041-4). Some companies (for example Eurogentec, Belgium) offer the preparation of such antibodies as an order service. Here, an increase in the amount of protein preferably means an increase in the amount of protein having an activity of a GFAT compared to corresponding not genetically modified wild-type plant cells or not genetically modified wild-type plants by at least 50%, in particular by at least 70%, preferably by at least 85% and particularly preferably by at least 100%. An increase in the amount of protein having an activity of a GFAT also means that plants or plant cells having no detectable amount of a protein having the activity of a GFAT have, after genetic modification according to the invention, a detectable amount of a protein having the activity of a GFAT.

The increased activity of a protein having the activity of a GFAT in plant extracts can be determined by methods known to the person skilled in the art as described, for example, in Samac et al. (2004, Applied Biochemistry and Biotechnology 113-116, Humana Press, Editor Ashok Mulehandani, 1167-1182, ISSN 0273-2289). A preferred method for determining the amount of the activity of a protein having the activity of a GFAT is given in General Methods, item 5.

An increased amount of (enzymatic) activity of proteins having the activity of a GFAT preferably means an increase of the activity of such proteins by at least 50%, preferably at least 70%, especially preferably by at least 85% and particularly preferably by at least 100% compared to corresponding not genetically modified wild-type plant cells or not genetically modified wild-type plants. An increase in the amount of enzymatic activity of proteins having the activity of a GFAT also means that plants or plant cells having no detectable amount of a protein having the activity of a GFAT have, after genetic modification according to the invention, a detectable amount of a protein having the activity of a GFAT.

In the context of the present invention, the term "genome" is to be understood as meaning the entire genetic material present in a plant cell. It is known to the person skilled in the art that, in addition to the nucleus, other compartments (for example plastids, mitochondria) also contain genetic material.

In the context of the present invention, the term "stably integrated nucleic acid molecule" is to be understood as meaning the integration of a nucleic acid molecule into the genome of the plant. A stably integrated nucleic acid molecule is characterized in that, during the replication of the corresponding integration site, it is multiplied together with the nucleic acid sequences of the host which border on the integration site, so that the integration site in the replicated DNA strand is surrounded by the same nucleic acid sequences as on the read strand which serves as a matrix for the replication.

A large number of techniques for stably integrating nucleic acid molecules into a plant host cell is available. These techniques include the transformation of plant cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as means of transformation, protoplast fusion, injection, electroporation of DNA, introduction of DNA by the biolistic approach and also further options (review in "Transgenic Plants", Leandro ed., Humana Press 2004, ISBN 1-59259-827-7).
The use of agrobacterium-mediated transformation of plant cells has been subject to in-depth studies and has been described exhaustively in EP 120516; Hoekema, IN: The Binary Plant Vector System Offsetdrukkerij Kanters B.V. Alblasserdam (1985), Chapter V; Fraley et al., Crit. Rev. Plant Sci. 4, 1-46 and in An et al. EMBO J. 4, (1985), 277-287. For the transformation of potatoes see, for example, Rocha-Sosa et al., EMBO J. 8, (1989), 29-33), for the transformation of tomato plants see, for example, US 5,565,347.

The transformation of monocotyledonous plants using vectors based on *Agrobacterium* transformation has been described, too (Chan et al., Plant Mol. Biol. 22, (1993), 491-506; Hiei et al., Plant J. 6, (1994) 271-282; Deng et al, Science in China 33, (1990), 28-34; Wilmink et al., Plant Cell Reports 11, (1992), 76-80; May et al., Bio/Technology 13, (1995), 486-492; Conner and Domisse, Int. J. Plant Sci. 153

(1992), 550-555; Ritchie et al, Transgenic Res. 2, (1993), 252-265). An alternative system for transforming monocotyledonous plants is the transformation using the biolistic approach (Wan and Lemaux, Plant Physiol. 104, (1994), 37-48; Vasil et al., Bio/Technology 11 (1993), 1553-1558; Ritala et al., Plant Mol. Biol. 24, (1994), 317-325; Spencer et al., Theor. Appl. Genet. 79, (1990), 625-631), the protoplast transformation, the electroporation of partially permeabilized cells, the introduction of DNA using glass fibers. In particular the transformation of corn has been described several times in the literature (cf., for example, WO95/06128, EP0513849, EP0465875, EP0292435; Fromm et al., Biotechnology 8, (1990), 833-844; Gordon-Kamm et al., Plant Cell 2, (1990), 603-618; Koziel et al., Biotechnology 11 (1993), 194-200; Moroc et al., Theor. Appl. Genet. 80, (1990), 721-726). The transformation of other grasses, such as, for example, switchgrass (*Panicum virgatum*) has also been described (Richards et al., 2001, Plant Cell Reporters 20, 48-54).

The successful transformation of other cereal species has also been described, for example for barley (Wan and Lemaux, s.o.; Ritala et al., s.o.; Krens et al., Nature 296, (1982), 72-74) and for wheat (Nehra et al., Plant J. 5, (1994), 285-297; Becker et al., 1994, Plant Journal 5, 299-307). All of the above methods are suitable in the context of the present invention.

Compared to the prior art, genetically modified plant cells according to the invention or genetically modified plants according to the invention offer the advantage that they produce higher amounts of hyaluronan than plants having only the activity of a hyaluronan synthase. This allows hyaluronan to be produced at little expense since the isolation of hyaluronan from plants having a higher hyaluronan content is less complicated and more cost efficient. Furthermore, compared to the plants described in the prior art, smaller cultivation areas are required to produce hyaluronan using the genetically modified plants according to the invention. This leads to the possibility to provide hyaluronan in sufficient amounts even for industrial application where it is currently not used owing to its scarcity and the high price. Virus-infected plant organisms of the genus *Chlorella* are unsuitable for producing relatively large amounts of hyaluronan. In the production of hyaluronan, virus-infected algae have the disadvantage that the genes required for hyaluronan synthesis are not stably

integrated into their genome (Van Etten and Meints, 1999, Annu. Rev. Microbiol. 53, 447-494), so that, for producing hyaluronan, the virus infection has to be repeated. Accordingly, it is not possible to isolate individual *Chlorella* cells which synthesize continuously the desired quality and quantity of hyaluronan. Furthermore, in virus-infected *Chlorella* algae, hyaluronan is only produced for a limited period of time, and as a result of the lysis caused by the virus, the algae are killed only about 8 hours after the infection (Van Etten et al., 2002, Arch Virol 147, 1479-1516). In contrast, the present invention offers the advantage that the genetically modified plant cells according to the invention and the genetically modified plants according to the invention can be propagated in an unlimited manner vegetatively or sexually and that they produce hyaluronan continuously.

The transgenic plants described in WO 05 012529, which have a nucleic acid molecule coding for a hyaluronan synthase, synthesize a relatively small amount of hyaluronan. In contrast, the present invention offers the advantage that genetically modified plant cells according to the invention and genetically modified plants according to the invention synthesize considerably higher amounts of hyaluronan.

Accordingly, the present invention also provides genetically modified plant cells according to the invention or genetically modified plants according to the invention which synthesize hyaluronan. Prefrably plant cells according to the invention or plants according to the invention synthesize at least 500 µg hyaluronan per gram fresh weight, with preference at least 1500 µg hyaluronan per gram fresh weight, particularly preferable at least 3500 µg hyaluronan per gram fresh weight, especially preferable at least 4000 µg hyaluronan per gram fresh weight an mostly preferable at least 5500 µg hyaluronan per gram fresh weight.

Prefrably plant cells according to the invention or plants according to the invention synthesize at most 25000 µg hyaluronan per gram fresh weight, with preference at most 20000 µg hyaluronan per gram fresh weight, particularly preferable at most 15000 µg hyaluronan per gram fresh weight, especially preferable at most 10000 µg hyaluronan per gram fresh weight an mostly preferable at most 6500 µg hyaluronan per gram fresh weight.

It has been observed that, over the development time, hyaluronan accumulates in

plant tissue; accordingly, the amount of hyaluronan with respect to the fresh weight or with respect to the dry weight in the genetically modified plant cells according to the invention or in the genetically modified plants according to the invention is to be determined with particular preference during harvesting or (one or two) days before harvesting of the plant cells in question or the plants in question. Here, use is made in particular of plant material (for example tubers, seeds, leaves) with respect to the amount of hyaluronan which is to be used for further processing.

Genetically modified plant cells according to the invention or genetically modified plants according to the invention which synthesize hyaluronan can be identified by isolating the hyaluronan that is synthesized by them and proving its structure.

Since plant tissue has the advantage that it does not contain hyaluronidases, a simple and rapid isolation method can be used for confirming the presence of hyaluronan in genetically modified plant cells according to the invention or genetically modified plants according to the invention. To this end, water is added to the plant tissue to be examined and the plant tissue is then comminuted mechanically (with the aid of, for example, a bead mill, a beater mill, a Warring blender, a juice extractor, etc.). If required, more water may then be added to the suspension, and cell debris and water-insoluble components are then removed by centrifugation or sieving. The presence of hyaluronan in the supernatant obtained after centrifugation can then be demonstrated using, for example, a protein which binds specifically to hyaluronan. A method for detecting hyaluronan with the aid of a protein that binds specifically to hyaluronan is described, for example, in US 5,019,498. Test kits (for example the hyaluronic acid (HA) test kit from Corgenix, Inc., Colorado, USA, Prod. No. 029-001; see also General Methods item 4). In parallel, it is possible to initially digest an aliquot of the centrifugation supernatant obtained with a hyaluronidase and then to confirm the presence of hyaluronan with the aid of a protein that specifically binds to hyaluronan, as described above. By the action of the hyaluronidase in the parallel batch, the hyaluronan present therein is degraded, so that after complete digestion it is no longer possible to detect significant amounts of hyaluronan.

The presence of hyaluronan in the centrifugation supernatant can furthermore also be confirmed using other analysis methods, such as, for example, IR, NMR or mass spectroscopy.

The overexpression, in corn, of a protein having the (enzymatic) activity of a GFAT fused translationally with a plastid signal peptide resulted in an increased UDP-glucosamine content, and the cytosolic overexpression, in corn, of a protein having the (enzymatic) activity of a GFAT resulted in an increased glucosamine 1-phosphate content in ground endosperm tissue. However, UDP-glucosamine and glucosamine 1-phosphate are not subtrates for the synthesis of hyaluronan by hyaluronan synthase. Furthermore, it is known that glucosamine has a cytotoxic effect on plant cells (Roberts et al., 1971, Plant Physiol. 48, 36-42) and that, if relatively high concentrations are present in plant cells, it is converted into glucosamine 6-phosphate. Glucosamine 6-phosphate is likewise toxic for plant cells. (WO 98 35047, US 6,444,878). Furthermore, it is known that proteins having the activity of a GFAT can be regulated in an inhibitory manner by metabolites which are formed in the further metabolic path for the synthesis of UDP-N-acetyl-glucosamine. Proteins having the activity of a GFAT, isolated form eucaryotes (both with animal and plant organisms) are inhibited, for example, by UDP-N-acetyl-glucosamine, which is one of the two substrates for hyaluronan synthase (Kornfeld, 1967, J. Biol. Chem. 242(13), 3135-3141; Graack et al., 2001, Biochem. J. 360, 401-412; Mayer et al., 1968, Plant Physiol. 43, 1097-1107). Bacterial proteins having the activity of a GFAT are inhibited by glucosamine 6-phosphate, a direct reaction product of the GFAT-catalyzed reaction (Deng et al., 2005, Metabolic Engineering 7, 201-214).

There are no indications in the literature what may limit the amount of hyaluronan synthesized in plant cells.

Accordingly, it has surprisingly been found that genetically modified plant cells or genetically modified plants having a nucleic acid molecule coding for a hyaluronan synthase and having additionally increased GFAT activity compared to genetically modified plant cells or genetically modified plants having (only) hyaluronan synthase activity produce significantly high amounts of hyaluronan.

In a preferred embodiment, the present invention relates to genetically modified plant cells according to the invention or genetically modified plants according to the invention, characterized in that they produce an increased amount of hyaluronan compared to genetically modified plant cells or compared to genetically modified

plants which (only) have the activity of a hyaluronan synthase or compared to genetically modified plant cells or compared to genetically modified plants having the activity of a hyaluronan synthase and no increased activity of a protein having the activity of a GFAT.

In the context of the present invention, the term "plant cell or plant (only) having the activity of a hyaluronan synthase" is to be understood as meaning a genetically modified plant cell or a genetically modified plant where the genetic modification consists in that it comprises a nucleic acid molecule coding for a hyaluronan synthase, compared to corresponding not genetically modified wild-type plant cells or not genetically modified wild-type plants.
In particular, "plant cells or plants (only) having the activity of a hyaluronan synthase" are characterized in that they synthesize hyaluronan and that they have no additional genetic modifications other than the introduction of a nucleic acid molecule coding for a hyaluronan synthase into not genetically modified wild-type plant cells or not genetically modified wild-type plants. Preferably, such plants do not have an increased activity of a protein having the activity of a GFAT.

The amount of hyaluronan produced by plant cells or plants can be determined with the aid of the methods which have already been described above, for example using a commercial test kit (for example the hyaluronic acid (HA) test kit from Corgenix, Inc., Colorado, USA, Prod. No. 029-001). A method which is preferred in the context of the present invention for determining the hyaluronan content in plant cells or plants is described under General Methods, item 4.

In a further embodiment of the present invention, the genetically modified plant cells according to the invention or the genetically modified plants according to the invention are plant cells of a green terrestrial plant or green terrestrial plants, respectively, which synthesize hyaluronan.

In the context of the present invention, the term "green terrestrial plant (Embryophyta)" is to be understood as defined in Strasburger, "Lehrbuch der Botanik" [Textbook of Botany], 34th ed., Spektrum Akad. Verl., 1999, (ISBN 3-8274-

0779-6).

A preferred embodiment of the present invention relates to genetically modified plant cells according to the invention of multicellular plants or genetically modified plants according to the invention which are multicellular organisms. Accordingly, this embodiment relates to plant cells or plants which do not originate from single-cell plants (protists) or which are not protists.

The genetically modified plant cells according to the invention or the genetically modified plants according to the invention may, in principle, be plant cells and plants, respectively, of any plant species, i.e. both monocotyledonous and dicotyledonous plants. They are preferably crop plants, i.e. plants cultivated by man for the purpose of feeding man and animal or for producing biomass and/or for preparing substances for technical, industrial purposes (for example corn, rice, wheat, alfalfa, rye, oats, barley, manioc, potato, tomato, switchgrass (*Panicum virgatum*), sago, mung beans, pas, sorghum, carrots, aubergine, radish, oilseed rape, soybeans, peanuts, cucumbers, pumpkins, melons, leek, garlic, cabbage, spinach, sweet potato, asparagus, courgettes, lettuce, artichokes, sweetcorn, parsnip, scorzonera, jerusalem artichoke, banana, sugarbeet, sugarcane, beetroot, broccoli, cabbage, onion, yellow beet, dandelion, strawberry, apple, apricot, plum, peach, grapevines, cauliflower, celery, bell peppers, swede, rhubarb). Particularly preferred are rice, tomato or potato plants.

In a preferred embodiment, the present invention relates to genetically modified plant cells according to the invention or genetically modified plants according to the invention where the nucleic acid molecule coding for hyaluronan synthase is characterized in that it codes for a viral hyaluronan synthase. The nucleic acid molecule coding for the hyaluronan synthase preferably codes for a hyaluronan synthase of a virus which infects algae.

With respect to an algae-infecting virus, the nucleic acid molecule which codes for a hyaluronan synthase preferably codes for a hyaluronan synthase of a *Chlorella*-infecting virus, particularly preferably a hyaluronan synthase of a *Paramecium bursaria Chlorella* Virus 1 and especially preferably a hyaluronan synthase of a

Paramecium bursaria Chlorella virus of an H1 strain.

In a further preferred embodiment, the present invention relates to genetically modified plant cells according to the invention or genetically modified plants according to the invention where the nucleic acid molecule which codes for the hyaluronan synthase is characterized in that the codons of the nucleic acid molecule coding for a hyaluronan synthase are modified compared to the codons of the nucleic acid molecule coding for the hyaluronan synthase of the organism that the hyaluronan synthase originates from. With particular preference, the codons of the hyaluronan synthase have been modified such that they are adapted to the frequency of the use of the codons of the plant cell or the plant into whose genome they are integrated or to be integrated.

Owing to the degeneration of the genetic code, amino acids can be encoded by one or more codons. In different organisms, the codons coding for an amino acid are used at different frequencies. Adapting the codon of a coding nucleic acid sequence to the frequency of their use in the plant cell or in the plant into whose genome the sequence to be expressed is to be integrated may contribute to an increased amount of translated protein and/or to the stability of the mRNA in question in the particular plant cells or plants. The frequency of use of codons in the plant cells or plants in question can be determined by the person skilled in the art by examining as many coding nucleic acid sequences of the organism in question as possible for the frequency with which certain codons are used for coding a certain amino acid. The frequency of the use of codons of certain organisms is known to the person skilled in the art and can be determined in a simple and rapid manner using computer programs. Suitable computer programs are publicly accessible and provided for free inter alia on the internet (for example http://gcua.schoedl.de/; http://www.kazusa.or.jp/codon/; http://www.entelechon.com/eng/cutanalysis.html). Adapting the codons of a coding nucleic acid sequence to the frequency of their use in the plant cell or in the plant into whose genome the sequence to be expressed is to be integrated can be carried out by *in vitro* mutagenesis or, preferably, by *de novo* synthesis of the gene sequence. Methods for the *de novo* synthesis of nucleic acid sequences are known to the person skilled in the art. A *de novo* synthesis can be

carried out, for example, by initially synthesizing individual nucleic acid oligonucleotides, hybridizing these with oligonucleotides complementary thereto, so that they form a DNA double strand, and then ligating the individual double-stranded oligonucleotides such that the desired nucleic acid sequence is obtained. The *de novo* synthesis of nucleic acid sequences including the adaptation of the frequency with which the codons are used to a certain target organism can also be sourced out to companies offering this service (for example Entelechon GmbH, Regensburg, Germany).

The nucleic acid molecule coding for the hyaluronan synthase is preferably characterized in that it codes for a hyaluronan synthase whose amino acid sequence is at least 70%, preferably at least 80%, with preference at least 90%, especially preferably at least 95% and most preferably at least 98% identical to the amino acid sequence shown under SEQ ID NO 2. In a particularly preferred embodiment, the nucleic acid molecule coding for the hyaluronan synthase is characterized in that it codes for a hyaluronan synthase having the amino acid sequence shown under SEQ ID No 2.

In a further embodiment, the nucleic acid molecule coding for a hyaluronan synthase is at least 70%, preferably at least 80%, with preference at least 90%, especially preferably at least 95% and most preferably at least 98% identical to the nucleic acid sequence shown under SEQ ID NO 1 or SEQ ID NO 3. In a particularly preferred embodiment, the nucleic acid molecule coding for the hyaluronan synthase is characterized in that it has the nucleic acid sequence shown under SEQ ID No 3.

On 8. 25. 2004, the plasmid IC 341-222, comprising a synthetic nucleic acid molecule coding for a *Paramecium bursaria Chlorella* virus hyaluronan synthase was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Brunswick, Germany, under the number DSM16664, in accordance with the Budapest treaty. The amino acid sequence shown in SEQ ID NO 2 can be derived from the coding region of the nucleic acid sequence integrated into the plasmid IC 341-222 and codes for a *Paramecium bursaria Chlorella* virus hyaluronan synthase.

Accordingly, the present invention also relates to genetically modified plant cells according to the invention or genetically modified plants according to the invention where the nucleic acid molecule which codes for the hyaluronan synthase is characterized in that it codes for a protein whose amino acid sequence can be derived from the coding region of the nucleic acid sequence inserted into plasmid DSM16664 or that it codes for a protein whose amino acid sequence is at least 70%, preferably at least 80%, with preference at least 90%, especially preferably at least 95% and most preferably at least 98% identical to the amino acid sequence which can be derived from the coding region of the nucleic acid sequence inserted into plasmid DSM16664.

The present invention also relates to genetically modified plant cells according to the invention or genetically modified plants according to the invention where the nucleic acid molecule coding for hyaluronan synthase is characterized in that it is the hyaluronan-synthase-encoding nucleic acid sequence integrated into plasmid DSM16664 or that it is at least 70%, preferably at least 80%, with preference at least 90%, especially preferably at least 95% and most preferably at least 98% identical to the nucleic acid sequence integrated into plasmid DSM16664.

The present invention furthermore relates to genetically modified plant cells according to the invention or genetically modified plants according to the invention which are characterized in that they have a foreign nucleic acid molecule stably integrated into their genome, said foreign nucleic acid molecule increasing the activity of a protein having the activity of a GFAT compared to corresponding not genetically modified wild-type plant cells or corresponding not genetically modified wild-type plants.

In the context of the present invention, the term "foreign nucleic acid molecule" is to be understood as meaning a molecule which either does not naturally occur in the corresponding wild-type plant cells or which does not naturally occur in the concrete spatial arrangement in wild-type plant cells or which is localized at a site in the genome of the wild-type plant cell where it does not naturally occur. Preferably, the

foreign nucleic acid molecule is a recombinant molecule comprising various elements whose combination or specific spatial arrangement does not naturally occur in plant cells.

In the context of the present invention, the term "recombinant nucleic acid molecule" is to be understood as meaning a nucleic acid molecule which has various nucleic acid molecules which are not naturally present in a combination like that present in a recombinant nucleic acid molecule. Thus, recombinant nucleic acid molecules may, in addition to nucleic acid molecules coding for a hyaluronan synthase and/or a protein having the activity of a GFAT, additionally have nucleic acid sequences which are not naturally present in combination with the nucleic acid molecules mentioned. The additional nucleic acid sequences mentioned which are present on a recombinant nucleic acid molecule in combination with a nucleic acid molecule encoding for a hyaluronan synthase or a protein having the activity of a GFAT may be any sequences. For example, they may be genomic plant nucleic acid sequences. The additional nucleic acid sequences are preferably regulatory sequences (promoters, termination signals, enhancers), particularly preferably regulatory sequences which are active in plant tissue, especially preferably tissue-specific regulatory sequences which are active in plant tissue. Methods for generating recombinant nucleic acid molecules are known to the person skilled in the art and comprise genetic engineering methods, such as, for example, linking of nucleic acid molecules by ligation, genetic recombination or the *de novo* synthesis of nucleic acid molecules (see, for example, Sambrok et al., Molecular Cloning, A Laboratory Manual, 3rd edition (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. ISBN: 0879695773, Ausubel et al., Short Protocols in Molecular Biology, John Wiley & Sons; 5th edition (2002), ISBN: 0471250929).

Genetically modified plant cells and genetically modified plants having a foreign nucleic acid molecule stably integrated into their genome or a plurality of foreign nucleic acid molecules stably integrated into their genome which code for hyaluronan synthase and which increase the activity of a protein having the activity of a GFAT compared to corresponding not genetically modified wild-type plant cells or not genetically modified wild-type plants can be distinguished from said wild-type plant

cells and said wild-type plants, respectively, inter alia by the fact that they comprise a foreign nucleic acid molecule which does not naturally occur in wild-type plant cells and wild-type plants, respectively, or in that such a molecule is integrated at a site in the genome of the genetically modified plant cell according to the invention or in the genome of the genetically modified plant according to the invention where it does not occur in wild-type plant cells and wild-type plants, respectively, i.e. in a different genomic environment. Furthermore, such genetically modified plant cells according to the invention and genetically modified plants according to the invention can be distinguished from not genetically modified wild-type plant cells and not genetically modified wild-type plants, respectively, in that they comprise at least one copy of the foreign nucleic acid molecule stably integrated into their genome, if appropriate in addition to copies of such a molecule naturally present in the wild-type plant cells or wild-type plants. If the foreign nucleic acid molecule(s) introduced into the genetically modified plant cells according to the invention or the genetically modified plant according to the invention are additional copies of molecules already naturally present in the wild-type plant cells or the wild-type plants, the genetically modified plant cells according to the invention and the genetically modified plants according to the invention can be distinguished from wild-type plant cells and wild-type plants, respectively, in particular by the fact that this additional copy/these additional copies is/are localized at sites in the genome where it/they is/are not present in wild-type plant cells and wild-type plants, respectively.

The stable integration of a nucleic acid molecule into the genome of a plant cell or a plant can be demonstrated by genetic methods and/or methods of molecular biology. A stable integration of a nucleic acid molecule into the genome of a plant cell or the genome of a plant is characterized in that in the progeny which has inherited said nucleic acid molecule, the stably integrated nucleic acid molecule is present in the same genomic environment as in the parent generation. The presence of a stable integration of a nucleic acid sequence in the genome of a plant cell or in the genome of a plant can be demonstrated using methods known to the person skilled in the art, inter alia with the aid of Southern blot analysis of the RFLP analysis (Restriction Fragment Length Polymorphism) (Nam et al., 1989, The Plant Cell 1, 699-705; Leister and Dean, 1993, The Plant Journal 4 (4), 745-750), with methods based on PCR, such as, for example, the analysis of differences in length in the amplified

fragments (Amplified Fragment Length Polymorphism, AFLP) (Castiglioni et al., 1998, Genetics 149, 2039-2056; Meksem et al., 2001, Molecular Genetics and Genomics 265, 207-214; Meyer et al., 1998, Molecular and General Genetics 259, 150-160) or using amplified fragments cleaved using restriction endonucleases (Cleaved Amplified Polymorphic Sequences, CAPS) (Konieczny and Ausubel, 1993, The Plant Journal 4, 403-410; Jarvis et al., 1994, Plant Molecular Biology 24, 685-687; Bachem et al., 1996, The Plant Journal 9 (5), 745-753).

In principle, the foreign nucleic acid molecule may be any nucleic acid molecule which increases, in the plant cell or plant, the activity of a protein having the activity of a GFAT.

In the context of the present invention, genetically modified plant cells according to the invention and genetically modified plants according to the invention can also be prepared by using insertion mutagenesis (review: Thorneycroft et al., 2001, Journal of experimental Botany 52 (361), 1593-1601). In the context of the present invention, insertion mutagenesis is to be understood as meaning in particular the insertion of transposons or transfer DNA (T-DNA) into a gene or into the vicinity of a gene coding for a protein having the activity of a GFAT, thus increasing the activity of a protein having the activity of a GFAT in the cell in question.

The transposons may either be transposons which occur naturally in the cell (endogenous transposons) or those which are not naturally present in said cell but were introduced into the cell by genetic engineering, such as, for example, transformation of the cell (heterologous transposons). The modification of the expression of genes by transposons is known to the person skilled in the art. A review of the use of endogenous and heterologous transposons as tools in plant biotechnology is given in Ramachandran and Sundaresan (2001, Plant Physiology and Biochemistry 39, 234-252).

T-DNA insertion mutagenesis is based on the fact that certain sections (T-DNA) of Ti plasmids from *Agrobacterium* can be integrated into the genome of plant cells. The site of integration into the plant chromosome is not predetermined, integration can be in any location. If the T-DNA is integrated into a section or into the vicinity of a section of the chromosome representing a gene function, this may result in an

increased gene expression and thus also a change in the activity of the protein encoded by the gene in question.

The sequences inserted into the genome (in particular transposons or T-DNA) are characterized in that they comprise sequences resulting in the activation of regulatory sequences of a gene coding for a protein having the activity of a GFAT ("activation tagging"). Preferably, the sequences inserted into the genome (in particular transposons or T-DNA) are characterized in that they are integrated into the vicinity of endogenous nucleic acid molecules in the genome of the plant cell or the plant coding for a protein having the activity of a GFAT.

Genetically modified plant cells according to the invention and genetically modified plants according to the invention can be generated, for example, using the method of activation tagging (see, for example, Walden et al., Plant J. (1991), 281-288; Walden et al., Plant Mol. Biol. 26 (1994), 1521-1528). This method is based on the activation of endogenous promoters by enhancer sequences, such as, for example, the enhancer of the 35S RNA promoter of the cauliflower mosaic virus or the octopine synthase enhancer.

In the context of the present invention, the term "T-DNA activation tagging" is to be understood as meaning a T-DNA fragment which comprises enhancer sequences and, by integration into the genome of a plant cell, increases the activity of a protein having the activity of a GFAT.

In the context of the present invention, the term "transposon activation tagging" is to be understood as meaning a transposon which comprises enhancer sequences and, by integration into the genome of a plant cell, increases the activity of a protein having the activity of a GFAT.

A preferred embodiment of the present invention relates to genetically modified plant cells according to the invention or genetically modified plants according to the invention which are characterized in that at least one foreign nucleic acid molecule codes for a protein having the (enzymatic) activity of a GFAT.

A particularly preferred embodiment of the present invention relates to genetically modified plant cells according to the invention or genetically modified plants according to the invention which are characterized in that a foreign nucleic acid molecule codes for a protein having the (enzymatic) activity of a GFAT.

According to the invention, the foreign nucleic acid molecule coding for a protein having the (enzymatic) activity of a GFAT may originate from any organism; preferably, said nucleic acid molecule originates from bacteria, fungi, animals, plants or viruses, particularly preferably from mammals, plants or bacteria and especially preferably from the mouse or *Escherichia coli.*

With respect to viruses, the foreign nucleic acid molecule coding for a protein having the (enzymatic) activity of a GFAT preferably originates from a virus which infects algae, with preference from a virus which infects algae of the genus *Chlorella*, particularly preferably from a *Paramecium bursaria Chlorella* virus and especially preferably from a *Paramecium bursaria Chlorella* virus of an H1 strain.

Instead of the naturally occurring nucleic acid molecule coding for a protein having the (enzymatic) activity of a GFAT, it is also possible for a nucleic acid molecule generated by mutagenesis to be introduced into the genetically modified plant cells according to the invention or the genetically modified plants according to the invention, where said mutagenized foreign nucleic acid molecule is characterized in that it codes for a protein having the (enzymatic) activity of a GFAT with reduced inhibition by metabolites (for example of the glucosamine metabolism). The preparation of such mutagenized nucleic acid molecules is described in an exemplary manner for a protein having the (enzymatic) activity of a GFAT from *Escherichia coli* in Deng et al. (2005, Metabolic Engineering 7, 201-214; WO 04 003175). Mutants for a protein having the activity of a GFAT from the mouse are described, for example, in Hu et al. (2004, J. Biol. Chem. 279 (29), 29988-29993).

Nucleic acid molecules coding for a protein having the activity of a GFAT are known to the person skilled in the art and described in the literature. Thus, nucleic acid molecules coding for a protein having the activity of a GFAT are described from viruses, for example for the *Chlorella* virus k2 (EMBL acc No AB107976.1), from

bacteria, for example for *Escherichia coli* (Dutka-Malen, 1988, Biochemie 70 (2), 287-290; EMBL acc No: L10328.1), from fungi, for example for *Saccharomyces cerevisiae* (EMBL acc No AF334737.1, Watzele et al., 1989, J. Biol. Chem. 264, 8753-8758), *Aspergillus niger* (EMBL acc No AY594332.1), *Candida albicans* (EMBL acc No X94753.1), from insects, for example for *Aedes aegyti* (Kato et al., *2002*, Insect. Biol. 11 (3), 207,216; EMBL acc No AF399922.1), *Drosophila melanogaster* (GFAT-1: EMBL acc No Y18627.1, GFAT-2: NCBI acc No NM_143360.2), from algae for *Volvariella volvacea* (EMBL acc No AY661466.1), from vertebrates for example for *Homo sapiens* (GFAT-1: EMBL acc No AF334737.1; GFAT-2: NCBI acc No BC000012.2, Oki et al., 1999, Genomics 57 (2),227-34), *Mus musculus* (GFAT-1: EMBL acc No AF334736.1; GFAT-2: EMBL acc No AB016780.1), or from plants for example for *Arabidopsis thaliana* (EMBL acc No AP001297.1; cds NCBI acc No BAB03027.1).

In a preferred embodiment, the present invention relates to genetically modified plant cells according to the invention and genetically modified plants according to the invention where the foreign nucleic acid molecule coding for a protein having the activity of a GFAT is selected from the group consisting of

a) nucleic acid molecules coding for a protein having the amino acid sequence given under SEQ ID NO 5 or a protein having the amino acid sequence given under SEQ ID NO 7 or a protein having the amino acid sequence given under SEQ ID NO 9;

b) nucleic acid molecules coding for a protein whose sequence is at least 60%, preferably at least 80%, with preference at least 90%, especially preferably at least 95% and most preferably at least 98% identical to the amino acid sequence given under SEQ ID NO 5, under SEQ ID NO 7 or under SEQ ID NO 9;

c) nucleic acid molecules comprising the nucleotide sequence shown under SEQ ID NO 4 or a sequence complementary thereto, the nucleotide sequence shown under SEQ ID NO 6 or a sequence complementary thereto, the nucleotide sequence shown under SEQ ID NO 8 or a sequence complementary thereto or the nucleotide sequence shown under SEQ ID NO 10 or a sequence complementary thereto;

d) nucleic acid molecules which are at least 70%, preferably at least 80%, with preference at least 90%, especially preferably at least 95% and most preferably at least 98% identical to the nucleic acid sequences described under a) or c);

e) nucleic acid molecules which hybridize under stringent conditions with at least one strand of the nucleic acid sequences described under a) or c);

f) nucleic acid molecules whose nucleotide sequence differs from the sequence of the nucleic acid molecules mentioned under a) or c) owing to the degeneration of the genetic code; and

g) nucleic acid molecules which are fragments, allelic variants and/or derivatives of the nucleic acid molecules mentioned under a), b), c), d), e) or f).

In the context of the present invention, the term "hybridization" means a hybridization under conventional hybridization conditions, preferably under stringent conditions, as described, for example, in Sambrock et al., Molecular Cloning, A Laboratory Manual, 2 ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). With particular preference, "hybridization" means a hybridization under the following conditions:

Hybridization buffer:

2xSSC; 10xDenhardt solution (Fikoll 400+PEG+BSA; ratio 1:1:1); 0.1% SDS; 5 mM EDTA; 50 mM Na2HPO4; 250 µg/ml of herring sperm DNA; 50 µg/ml of tRNA; or

25 M sodium phosphate buffer pH 7.2; 1 mM EDTA; 7% SDS

Hybridization temperature:

T=65 to 68°C

Wash buffer: 0.1xSSC; 0.1% SDS

Wash temperature: T=65 to 68°C.

Nucleic acid molecules which hybridize with nucleic acid molecules coding for a protein having the activity of a GFAT may originate from any organism; accordingly, they may originate from bacteria, fungi, animals, plants or viruses.

Nucleic acid molecules hybridizing with nucleic acid molecules coding for protein having the activity of a GFAT particularly preferably originate from mammals, plants

or bacteria and especially preferably from the mouse or *Escherichia coli*.

Nucleic acid molecules hybridizing with nucleic acid molecules coding for protein having the activity of a GFAT-1 or a GFAT-2 preferably originate from a eucaryotic organism, particularly preferably they originate from an animal organism, especially preferably from the mouse.

Nucleic acid molecules which hybridize with the molecules mentioned may be isolated, for example, from genomic or from cDNA libraries. Such nucleic acid molecules can be identified and isolated using the nucleic acid molecules mentioned or parts of these molecules or the reverse complements of these molecules, for example by hybridization according to standard methods (see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2 ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) or by amplification using PCR.

As hybridization sample for isolating a nucleic acid sequence coding for a protein having the activity of a GFATor the activity of a GFAT-1 or the activity of a GFAT-2, it is possible to use, for example, nucleic acid molecules having exactly or essentially the nucleotide sequence given under SEQ ID NO 4 or under SEQ ID NO 6 or under SEQ ID NO 8 or under SEQ ID NO 10, or parts of these sequences.

The fragments used as hybridization samples may also be synthetic fragments or oligonucleotides prepared using the customary synthesis techniques, whose sequence is essentially identical to the nucleic acid molecule described in the context of the present invention. Once genes which hybridize with the nucleic acid sequences described in the context of the present invention are identified and isolated, the sequence should be determined and the properties of the proteins coded for by this sequence should be analyzed to determine whether they are proteins having the activity of a GFAT. Methods of how to determine whether a protein has the activity of a protein having the activity of a GFAT (for example Mayer et al., 1968, Plant Physiol. 43, 1097-1107; Deng et al., 2005, Metabolic Engineering 7, 201-214), a GFAT-1 or a GFAT-2 (for example Hu et al., 2004, J. Biol. Chem. 279 (29), 29988-29993) are known to the person skilled in the art and described, inter alia, in the literature mentioned.

The molecules hybridizing with the nucleic acid molecules described in the context of the present invention comprise in particular fragments, derivatives and allelic variants of the nucleic acid molecules mentioned. In the context of the present invention, the

term "derivative" means that the sequences of these molecules differ in one or more positions from the sequences of the nucleic acid molecules described above and are highly identical to these sequences. The differences to the nucleic acid molecules described above may, for example, be due to deletion (in particular 5′- and/or 3′-deletions, leading to N- and/or C-terminal deletions of the corresponding protein), addition, substitution, insertion or recombination.

In the context of the present invention, the term "identity" means a sequence identity over the entire length of the coding region of a nucleic acid molecule or the entire length of an amino acid sequence coding for a protein of at least 60%, in particular in identity of at least 70%, preferably of at least 80%, particularly preferably of at least 90% and especially preferably of at least 95% and most preferably at least 98%. In the context of the present invention, the term "identity" is to be understood as meaning the number of identical amino acids/nucleotides (identity) with other proteins/nucleic acids, expressed in percent. Preferably, the identity with respect to a protein having the activity of a GFAT is determined by comparison with the amino acid sequence given under SEQ ID NO 5 or SEQ ID NO 7 or SEQ ID NO 9 and the identity with respect to a nucleic acid molecule coding for a protein having the activity of a GFAT is determined by comparison with the nucleic acid sequence given under SEQ ID NO 4 or SEQ ID NO 6 or SEQ ID NO 8 or SEQ ID NO 10 with other proteins/nucleic acids with the aid of computer programs. If sequences to be compared with one another are of different lengths, the identity is to be determined by determining the identity in percent of the number of amino acids which the shorter sequence shares with the longer sequence. Preferably, the identity is determined using the known and publicly available computer program ClustalW (Thompson et al., Nucleic Acids Research 22 (1994), 4673-4680). ClustalW is made publicly available by Julie Thompson (Thompson@EMBL-Heidelberg.DE) and Toby Gibson (Gibson@EMBL-Heidelberg.DE), European Molecular Biology Laboratory, Meyerhofstrasse 1, D 69117 Heidelberg, Germany. ClustalW can also be downloaded from various internet pages, inter alia from IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire, B.P.163, 67404 Illkirch Cedex, France; ftp://ftp-igbmc.u-strasbg.fr/pub/) and from EBI (ftp://ftp.ebi.ac.uk/pub/software/) and all mirrored internet pages of the EBI (European Bioinformatics Institute, Wellcome Trust

Genome Campus, Hinxton, Cambridge CB10 1SD, UK).

Preferably, use is made of the ClustalW computer program of version 1.8 to determine the identity between proteins described in the context of the present invention and other proteins. Here, the parameters have to be set as follows: KTUPLE=1, TOPDIAG=5, WINDOW=5, PAIRGAP=3, GAPOPEN=10, GAPEXTEND=0.05, GAPDIST=8, MAXDIV=40, MATRIX=GONNET, ENDGAPS(OFF), NOPGAP, NOHGAP.

Preferably, use is made of the ClustalW computer program of version 1.8 to determine the identity for example between the nucleotide sequence of the nucleic acid molecules described in the context of the present invention and the nucleotide sequence of other nucleic acid molecules. Here, the parameters have to be set as follows:

KTUPLE=2, TOPDIAGS=4, PAIRGAP=5, DNAMATRIX:IUB, GAPOPEN=10, GAPEXT=5, MAXDIV=40, TRANSITIONS: unweighted.

Identity furthermore means that there is a functional and/or structural equivalence between the nucleic acid molecules in question or the proteins encoded by them. The nucleic acid molecules which are homologous to the molecules described above and represent derivatives of these molecules are generally variations of these molecules which represent modifications having the same biological function. They may be either naturally occurring variations, for example sequences from other species, or mutations, where these mutations may have occurred in a natural manner or were introduced by targeted mutagenesis. Furthermore, the variations may be synthetically produced sequences. The allelic variants may be either naturally occurring variants or synthetically produced variants or variants generated by recombinant DNA techniques. A special form of derivatives are, for example, nucleic acid molecules which differ from the nucleic acid molecules described in the context of the present invention owing to the degeneration of the genetic code.

The various derivatives of the nucleic acid molecules coding for a protein having the activity of a GFAT have certain common characteristics.

These may, for example, be biological activity, substrate specificity, molecular weight, immunological reactivity, conformation, etc., and also physical properties, such as, for

example, the mobility properties in gel electrophoresis, chromatographic behavior, sedimentation coefficients, solubility, spectroscopic properties, stability, pH optimum, temperature optimum, etc. Preferred properties of proteins having the activity of a GFAT are known to the person skilled in the art, have already been mentioned above and are to apply here in an analogous manner.

In a further preferred embodiment, the present invention relates to genetically modified plant cells according to the invention or genetically modified plants according to the invention where nucleic acid molecules coding for a protein having the (enzymatic) activity of a GFAT are characterized in that the codons of said nucleic acid molecules are different from the codons of the nucleic acid molecules which code for said protein having the (enzymatic) activity of a GFAT of the parent organism. Particularly preferably, the codons of the nucleic acid molecules coding for a protein having the (enzymatic) activity of a GFAT are changed thus that they are adapted to the frequency of use of the codons of the plant cell or the plant into whose genome they are integrated or to be integrated.

The present invention furthermore provides genetically modified plant cells according to the invention or genetically modified plants according to the invention characterized in that the foreign nucleic acid molecules stably integrated into the genome of the plant cell or the plant encoding for a hyaluronan synthase and/or coding for a protein having the (enzymatic) activity of a GFAT are linked to regulatory elements initiating the transcription in plant cells (promoters). These may be homologous or heterologous promoters. The promoters can be constitutive, tissue-specific, development-specific or regulated by external factors (for example after application of chemical substances, by action of abiotic factors, such as heat and/or cold, draught, disease, etc.). Here, nucleic acid molecules coding for a hyaluronan synthase or a protein having the (enzymatic) activity of a GFAT, which nucleic acid molecules are integrated into the genome of a genetically modified plant cell according to the invention or a genetically modified plant according to the invention, may in each case be linked to the same promoter, or the individual sequences may be linked to different promoters.

A preferred embodiment of the present invention relates to genetically modified plant cells according to the invention or genetically modified plants according to the invention where at least one foreign nucleic acid molecule, particularly preferably at least two foreign nucleic acid molecules, especially preferably three foreign nucleic acid molecules selected from the group consisting of nucleic acid molecules coding for a hyaluronan synthase or a protein having the (enzymatic) activity of a GFAT is (are) linked to a tissue-specific promoter. Preferred tissue-specific promoters are promoters which initiate transcription specifically in plant tuber, fruit or seed cells or leaves.

To express nucleic acid molecules coding for a hyaluronan synthase or a protein having the (enzymatic) activity of a GFAT, these are preferably linked to regulatory DNA sequences ensuring the transcription in plant cells. These include in particular promoters. In general, any promoter active in plant cells is suitable for the expression.

Here, the promoter may be chosen such that expression is constitutively or only in a certain tissue, at a certain point of the development of the plant or at a point of time determined by external factors. Both in respect to the plant and in respect of the nucleic acid molecule to be expressed, the promoter may be homologous or heterologous.

Suitable promoters are, for example, the promoter of 35S RNS of the cauliflower mosaic virus or the ubiquitin promoter from corn or the *Cestrum* YLCV (Yellow Leaf Curling Virus; WO 01 73087; Stavolone et al., 2003, Plant Mol. Biol. 53, 703-713) for a constitutive expression, the patatingen promoter B33 (Rocha-Sosa et al., EMBO J. 8 (1989), 23-29) for a tuber-specific expression in potatoes or a fruit-specific promoter for tomato, such as, for example, the polygalacturonase promoter from tomato (Montgomery et al., 1993, Plant Cell 5, 1049-1062) or the E8 promoter from tomato (Metha et al., 2002, Nature Biotechnol. 20(6), 613-618) or the ACC oxidase promoter from peach (Moon and Callahan, 2004, J. Experimental Botany 55 (402), 1519-1528) or a promoter which ensures expression only in photosynthetically active tissues, for example the ST-LS1 promoter (Stockhaus et al., Proc. Natl. Acad. Sci. USA 84 (1987), 7943-7947; Stockhaus et al., EMBO J. 8 (1989), 2445-2451) or for an endosperm-specific expression the HMWG promoter from wheat, the USP

promoter, the phaseolin promoter, promoters of zein genes from corn (Pedersen et al., Cell 29 (1982), 1015-1026; Quatroccio et al., Plant Mol. Biol. 15 (1990), 81-93), the glutelin promoter (Leisy et al., Plant Mol. Biol. 14 (1990), 41-50; Zheng et al., Plant J. 4 (1993), 357-366; Yoshihara et al., FEBS Lett. 383 (1996), 213-218), the shrunken-1 promoter (Werr et al., EMBO J. 4 (1985), 1373-1380), a globulin promoter (Nakase et al., 1996, Gene 170(2), 223-226) or a prolamin promoter (Qu und Takaiwa, 2004, Plant Biotechnology Journal 2(2), 113-125). However, it is also possible to use promoters which are only active at a point in time determined by external factors (see, for example, WO 9307279). Of particular interest here may be promoters of heat-shock proteins which permit a simple induction. It is furthermore possible to use seed-specific promoters, such as, for example, the USP promoter from *Vicia faba* which ensures a seed-specific expression in *Vicia faba* and other plants (Fiedler et al., Plant Mol. Biol. 22 (1993), 669-679; Bäumlein et al., Mol. Gen. Genet. 225 (1991), 459-467).

The use of promoters present in the genome of algae-infecting viruses are also suitable for expressing nucleic acid sequences in plants (Mitra et al., 1994, Biochem. Biophys Res Commun 204(1), 187-194; Mitra and Higgins, 1994, Plant Mol Biol 26(1), 85-93, Van Etten et al., 2002, Arch Virol 147, 1479-1516).

In the context of the present invention, the term "tissue specific" is to be understood as meaning the substantial limitation of a manifestation (for example initiation of transcription) to a certain tissue.

In the context of the present invention, the terms "tuber, fruit or seed cell" are to be understood as meaning all cells present in a tuber, a fruit or in a seed.

In the context of the present invention, the term "homologous promoter" is to be understood as meaning a promoter which is naturally present in plant cells or plants used for the preparation of genetically modified plant cells according to the invention or genetically modified plants according to the invention (homologous with respect to the plant cell or the plant) or as meaning a promoter which regulates the regulation of the expression of a gene in the organism from which the sequence was isolated (homologous with respect to the nucleic acid molecule to be expressed).

In the context of the present invention, the term "heterologous promoter" is to be understood as meaning a promoter which is not naturally present in plant cells or plants used for the preparation of genetically modified plant cells according to the invention or genetically modified plants according to the invention (heterologous with respect to the plant cell or plant) or as meaning a promoter which is, in the organism from which a nucleic acid sequence to be expressed was isolated, not naturally present for regulating the expression of said nucleic acid sequence (heterologous with respect to the nucleic acid molecule to be expressed).

Also present may be a termination sequence (polyadenylation signal) which serves to add a poly-A tail to the transcript. The poly-A tail is thought to act in stabilizing the transcripts. Such elements are described in the literature (cf. Gielen et al., EMBO J. 8 (1989), 23-29) and can be exchanged as desired.

It is also possible for intron sequences to be present between the promoter and the coding region. Such intron sequences may lead to stability of expression and in increased expression in plants (Callis et al., 1987, Genes Devel. 1, 1183-1200; Luehrsen, and Walbot, 1991, Mol. Gen. Genet. 225, 81-93; Rethmeier et al., 1997; Plant Journal 12(4), 895-899; Rose and Beliakoff, 2000, Plant Physiol. 122 (2), 535–542; Vasil et al., 1989, Plant Physiol. 91, 1575-1579; XU et al., 2003, Science in China Series C Vol.46 No.6, 561-569). Suitable intron sequences are, for example, the first intron of the sh1 gene from corn, the first intron of the poly-ubiquitin gene 1 from corn, the first intron of the EPSPS gene from rice or one of the first two introns of the PAT1 gene from *Arabidopsis*.

The present invention also relates to plants comprising genetically modified plant cells according to the invention. Such plants may be produced by regeneration from genetically modified plant cells according to the invention.

The present invention also relates to processible or consumable parts of genetically modified plants according to the invention comprising genetically modified plant cells according to the invention.

In the context of the present invention, the term "processible parts" is to be understood as meaning plant parts which are used for preparing foodstuff or feedstuff, which are used as a raw material source for industrial processes, as a raw material source for the preparation of pharmaceutical products or as a raw material source for the preparation of cosmetic products.

In the context of the present invention, the term "consumable parts" is to be understood as meaning plant parts which serve as food for man or are used as animal feed.

The present invention also relates to a propagation material of genetically modified plants according to the invention comprising a genetically modified plant cell according to the invention.

Here, the term "propagation material" comprises those components of the plant which are suitable for generating progeny via the vegetative or generative route. Suitable for vegetative propagation are, for example, cuttings, callus cultures, rhizomes or tubers. Other propagation material includes, for example, fruits, seeds, seedling, protoplasts, cell cultures, etc. The propagation material preferably takes the form of tubers, fruits or seeds.

In a further embodiment, the present invention relates to harvestable plant parts of genetically modified plants according to the invention, such as fruits, storage and other roots, flowers, buds, shoots, leaves or stalks, preferably seeds, fruits or tubers, these harvestable parts comprising genetically modified plant cells according to the invention.

Preferably, the present invention relates to propagation material according to the invention or harvestable parts of plants according to the invention comprising hyaluronan. Particularly preferred is propagation material according to the invention or harvestable parts of plants according to the invention which synthesize hyaluronan.

In the context of the present invention, the term "potato plant" or potato" is to be understood as meaning plant species of the genus *Solanum*, particularly tuber-producing species of the genus *Solanum* and in particular *Solanum tuberosum.*

In the context of the present invention, the term "tomato plant" or "tomato" is to be understood as meaning plant species of the genus *Lycopersicon*, in particular *Lycopersicon esculentum.*

The further advantage of the present invention is that harvestable parts, propagation material, processible parts or consumable parts of genetically modified plants according to the invention comprise more hyaluronan than hyaluronan-synthesizing transgenic plants described in the literature. Accordingly, genetically modified plants according to the invention are not only particularly suitable for use as raw material from which hyaluronan may be isolated but can also be used directly as foodstuff/feedstuff or for preparing foodstuff/feedstuff having a prophylactic or therapeutic character (for example for osteoarthritis prophylaxis, US 6,607,745). Since genetically modified plants according to the invention have a higher hyaluronan content than the plants described in the literature, the preparation of such foodstuff/feedstuff requires lower amounts of harvestable parts, propagation material, processible parts or consumable parts of genetically modified plants according to the invention. If consumable parts of genetically modified plants according to the invention are consumed, for example, directly as a so-called "nutraceutical", it is possible to achieve a positive effect even by ingesting relatively small amounts of substance. This may be of particular significance inter alia in the production of animal feed, since animal feed having too high a content of plant components is unsuitable as feedstuff for various animal species.

By virtue of the high capacity of hyaluronan to bind water, harvestable parts, propagation material, processible parts or consumable parts of genetically modified plants according to the invention furthermore have the advantage that less thickeners are required when solidified foodstuff/feedstuff is produced. Thus, for example, the production of jelly requires less sugar, which is associated with an additional positive effect on health. In the production of foodstuff/feedstuff requiring the dehydration of

the crude plant material, the advantage of using harvestable parts, propagation material, processible parts or consumable parts of genetically modified plants according to the invention consists in the fact that less water has to be removed from the plant material in question, resulting in lower production costs and, owing to more gentle preparation methods (for example lower and/or shorter input of heat), an elevated nutritional value of the foodstuff/feedstuff in question. Thus, for example, in the production of tomato ketchup less energy has to be introduced in order to achieve the desired consistency.

The present invention furthermore provides a process for preparing a plant which synthesizes hyaluronan, which comprises

a) genetically modifying a plant cell, where the genetic modification comprises steps i to ii below

   i) introduction of a foreign nucleic acid molecule encoding for a hyaluronan synthase into the plant cell

   ii) introduction of a genetic modification into the plant cell, the genetic modification resulting in an increase of the activity of a protein having the (enzymatic) activity of a GFAT compared to corresponding not genetically modified wild-type plant cells

where steps i to ii can be carried out in any order, individually, or any combinations of steps i to ii can be carried out simultaneously

b) regenerating a plant from plant cells from step a);

c) generating, if appropriate, further plants using the plants according to step b), where, if appropriate, plant cells are isolated from plants according to step b) and the process steps a) to c) are repeated until a plant is generated which has a foreign nucleic acid molecule coding for a hyaluronan synthase and has an increased activity of a protein having the (enzymatic) activity of a GFAT compared to corresponding not genetically modified wild-type plant cells.

The present invention preferably relates to processes for preparing a plant which synthesizes hyaluronan which comprises

a) genetically modifying a plant cell, where the genetic modification comprises steps i to ii below in any order, or any combinations of steps i to ii may be

carried out individually or simultaneously,

i) introduction of a foreign nucleic acid molecule encoding for a hyaluronan synthase into the plant cell

ii) introduction of a genetic modification into the plant cell, the genetic modification resulting in an increase of the activity of a protein having the (enzymatic) activity of a GFAT compared to corresponding not genetically modified wild-type plant cells

b) regenerating a plant from plant cells comprising the genetic modification according to steps

i) a) i

ii) a) ii

iii) a) i and a) ii,

c) introducing into plant cells of plants according to step

i) b) i a genetic modification according to step a) ii,

ii) b) ii a genetic modification according to step a) i,

and regenerating a plant

d) generating, if appropriate, further plants with the aid of the plants obtained according to any of steps b) iii or c) i or c) ii.

The genetic modifications introduced according to step a) into the plant cell may in principle be any type of modification resulting in an increased activity of a protein having the (enzymatic) activity of a GFAT.

The regeneration of the plants according to step b) and, if appropriate, step c) of the processes according to the invention can be carried out using methods known to the person skilled in the art (described, for example, in "Plant Cell Culture Protocols", 1999, edited by R.D. Hall, Humana Press, ISBN 0-89603-549-2).

The generation of further plants (depending on the process according to step c) or step d)) of the processes according to the invention can be carried out, for example, by vegetative propagation (for example via cuttings, tubers or via callus culture and regeneration of intact plants) or via generative propagation. In this context, generative propagation generally takes place under controlled conditions, i.e.

selected plants with specific characteristics are hybridized with one another and multiplied. The generation preferably takes place in such a manner that the further plants (depending on the process generated according to step c) or step d)) comprise the modifications introduced in the preceding steps.

In processes according to the invention for preparing plants which synthesize hyaluronan, the genetic modifications for generating the genetically modified plant cells according to the invention can be carried out simultaneously or in successive steps. Here, it is immaterial whether the same method as for the genetic modification introducing a foreign nucleic acid molecule coding for a hyaluronan synthase into the plant cell is used for successive genetic modifications resulting in an increased activity of a protein having the (enzymatic) activity of a GFAT.

In a further embodiment of processes according to the invention for preparing a plant which synthesizes hyaluronan, the genetic modification consists in the introduction of at least one foreign nucleic acid molecule into the genome of the plant cell, where the presence or the expression of the foreign nucleic acid molecule(s) results in an increased activity of a protein having the (enzymatic) activity of a GFAT in the plant cell.

As already described above for the foreign nucleic acid molecules introduced for genetic modification into the plant cell or plant, what is introduced in step a) of the processes according to the invention for preparing a plant which synthesizes hyaluronan may be an individual nucleic acid molecule or a plurality of nucleic acid molecules. Thus, the foreign nucleic acid molecules coding for a hyaluronan synthase and/or coding for a protein having the (enzymatic) activity of a GFAT may be present together on a single nucleic acid molecule, or they may be present on separate nucleic acid molecules. If the nucleic acid molecules coding for a hyaluronan synthase and coding for a protein having the activity of a GFAT are present on a plurality of nucleic acid molecules, these nucleic acid molecules may be introduced simultaneously or in successive steps into a plant cell.

Furthermore, to introduce a foreign nucleic acid molecule in the practice of processes

according to the invention for preparing a plant which synthesizes hyaluronan, it is possible to use, instead of a wild-type plant cell or wild-type plant, mutant cells or mutants which are distinguished in that they already have an increased activity of a protein having the (enzymatic) activity of a GFAT. If the mutant cell or the mutant already has an increased activity of a protein having the (enzymatic) activity of a GFAT compared to the corresponding wild-type plant cells or wild-type plants, it is sufficient for carrying out a process according to the invention for producing a plant which synthesizes hyaluronan to introduce into said mutant cell or mutant a foreign nucleic acid molecule coding for a hyaluronan synthase.

All said further above concerning the use of mutants for the preparation of genetically modified plant cells according to the invention or genetically modified plants according to the invention applies here in an analogous manner.

In preferred embodiments, the present invention relates to processes according to the invention for producing a plant which synthesizes hyaluronan, wherein the nucleic acid molecule coding for a hyaluronan synthase in step a) is selected from the group consisting of:

a) nucleic acid molecules characterized in that they code for a viral hyaluronan synthase,

b) nucleic acid molecules characterized in that they code for a hyaluronan synthase of a *Chlorella*-infecting virus,

c) nucleic acid molecules characterized in that they code for a hyaluronan synthase of a *Paramecium bursaria Chlorella* Virus 1,

d) nucleic acid molecules characterized in that they code for a hyaluronan synthase of a *Paramecium bursaria Chlorella* Virus 1 of strain H1,

e) nucleic acid molecules characterized in that the codons of the nucleic acid molecule coding for a hyaluronan synthase are modified compared to the codons of the nucleic acid molecule which codes for the hyaluronan synthase in the parent organism of the hyaluronan synthase,

f) nucleic acid molecules characterized in that the codons of the hyaluronan synthase have been modified thus that they are adapted to the frequency of the use of the codons of the plant cell or of the plant into whose genome they are to be integrated or are integrated,

g) nucleic acid molecules characterized in that they code for a hyaluronan synthase having the amino acid sequence shown under SEQ ID NO 2 or that they code for a hyaluronan synthase whose amino acid sequence is at least 70%, preferably at least 80%, particularly preferably at least 90%, especially preferably at least 95% and and most preferably at least 98% identical to the amino acid sequence shown under SEQ ID NO 2,

h) nucleic acid molecules characterized in that they code for a protein whose amino acid sequence can be derived from the coding region of the nucleic acid sequence inserted into plasmid DSM16664 or that it codes for a protein whose amino acid sequence is at least 70%, preferably at least 80%, particularly preferably at least 90%, especially preferably at least 95% and most preferably at least 98% identical to the amino acid sequence which can be derived from the coding region of the nucleic acid sequence inserted into plasmid DSM16664,

i) nucleic acid molecules comprising a nucleic acid sequence shown under SEQ ID NO 1 or SEQ ID NO 3 or being at least 70%, preferably at least 80%, with preference at least 90%, especially preferably at least 95% and most preferably at least 98% identical to the nucleic acid sequence shown under SEQ ID NO 1 or SEQ ID NO 3,

j) nucleic acid molecules comprising the nucleic acid sequence inserted into plasmid DSM16664 or being at least 70%, preferably at least 80%, with preference at least 90%, especially preferably at least 95% and most preferably at least 98% identical to the nucleic acid sequence inserted into plasmid DSM16664,

k) nucleic acid molecules coding for a hyaluronan synthase, where the nucleic acid sequences coding for the hyaluronan synthase are linked to regulatory elements (promoter) which initiate the transcription in plant cells or

l) nucleic acid molecules according to k) where the promoters are tissue-specific promoters, particularly preferably promoters which initiate the initiation of transcription specifically in plant tuber, fruit or seed cells.

In preferred embodiments, the present invention relates to processes according to the invention for producing a plant which synthesizes hyaluronan, where the nucleic

acid molecule coding for a protein having the activity of a GFAT is selected from the group consisting of:

a) nucleic acid molecules characterized in that they code for a protein having the activity of a GFAT originating from bacteria, animals or plants, preferably from *Escherichia coli* or the mouse,

b) nucleic acid molecules characterized in that they code for a protein having the activity of a GFAT of a *Chlorella*-infecting virus,

c) nucleic acid molecules characterized in that they code for a protein having the activity of a GFAT of a *Paramecium bursaria Chlorella* virus,

d) nucleic acid molecules characterized in that the codons of the nucleic acid molecule coding for a protein having the activity of a GFAT are modified compared to the codons of a nucleic acid molecule coding for the corresponding protein having the activity of a GFAT of the parent organism,

e) nucleic acid molecules characterized in that the codons of the protein having the activity of a GFAT are modified thus that they are adapted to the frequency of the use of the codons of the plant cell or of the plant into whose genome they are to be integrated or are integrated,

f) nucleic acid molecules coding for a protein having the amino acid sequence shown under SEQ ID NO 5 or for a protein having the amino acid sequence shown under SEQ ID NO 7 or for a protein having the amino acid sequence shown under SEQ ID NO 9;

g) nucleic acid molecules coding for a protein whose sequence is at least 70%, preferably at least 80%, with preference at least 90%, especially preferably at least 95% and most preferably at least 98% identical to the amino acid sequence shown under SEQ ID NO 5 or under SEQ ID NO 7 or under SEQ ID NO 9;

h) nucleic acid molecules comprising the nucleic acid sequence shown under SEQ ID NO 4 or a sequence complementary thereto or the nucleic acid sequence shown under SEQ ID NO 6 or a sequence complementary thereto or the nucleic acid sequence shown under SEQ ID NO 8 or a sequence complementary thereto or the nucleic acid sequence shown under SEQ ID NO 10 or a sequence complementary thereto;

i) nucleic acid molecules which are at least at least 70%, preferably at least 80%,

with preference at least 90%, especially preferably at least 95% and most preferably at least 98% identical to the nucleic acid sequences described under h);

j) nucleic acid molecules which hybridize under stringent conditions with at least one strand of the nucleic acid sequences described under f) or h);

k) nucleic acid molecules whose nucleotide sequence differs from the sequence of the nucleic acid molecules mentioned under f) or h) owing to the degeneration of the genetic code; and

l) nucleic acid molecules which are fragments, allelic variants and/or derivatives of the nucleic acid molecules mentioned under a), b), c), d), e), f) or h),

m) nucleic acid molecules coding for a protein having the activity of a GFAT, where the nucleic acid sequences coding for a protein having the activity of a GFAT are linked to regulatory elements (promoter) which initiate the transcription in plant cells or

n) nucleic acid molecules according to m), where the promoters are tissue-specific promoters, particularly preferably promoters which initiate the transcription specifically in plant tuber, leaf, fruit or seed cells.

In a further preferred embodiment, processes according to the invention for producing a plant which synthesizes hyaluronan are used for producing genetically modified plants according to the invention.

The present invention also provides plants obtainable by a process according to the invention for producing a plant which synthesizes hyaluronan.

The present invention furthermore relates to a process for producing hyaluronan which comprises the step of extracting hyaluronan from genetically modified plant cells according to the invention, from genetically modified plants according to the invention, from propagation material according to the invention, from harvestable plant parts according to the invention or from plants or parts of these plants obtainable by a process according to the invention for producing plants which synthesize hyaluronan.

Preferably, such a process also comprises the step of harvesting the cultivated genetically modified plant cells according to the invention, the genetically modified plants according to the invention, the propagation material according to the invention, the harvestable plant parts according to the invention, the processible plant parts according to the invention prior to extracting the hyaluronan, and particularly preferably furthermore the step of cultivating genetically modified plant cells according to the invention or genetically modified plants according to the invention prior to harvesting.

In contrast to bacterial or animal tissues, plant tissues have no hyaluronidases and do not contain any hyaladherins. Accordingly, as already described above, extraction of hyaluronan from plant tissues is possible using relatively simple methods. If required, the aqueous extracts, described above, of plant cells or tissues containing hyaluronan can be purified further using methods known to the person skilled in the art, such as, for example, repeated precipitation with ethanol. A preferred method for purifying hyaluronan is described under General Methods item 2.

The processes already described for extracting hyaluronan from genetically modified plant cells according to the invention or genetically modified plants according to the invention are also suitable for isolating hyaluronan from propagation material according to the invention, from harvestable plant parts according to the invention or from plants or parts of these plants obtainable by a process according to the invention for preparing plants which synthesize hyaluronan.

The present invention also provides the use of genetically modified plant cells according to the invention, genetically modified plants according to the invention, propagation material according to the invention, harvestable plant parts according to the invention, processible plant parts according to the invention or plants obtainable by a process according to the invention for preparing hyaluronan.

The present invention furthermore relates to compositions comprising genetically modified plant cells according to the invention. Here, it is immaterial whether the plant cells are intact or no longer intact because they have been destroyed, for

example, by processing. The compositions are preferably foodstuff or feedstuff, pharmaceutical or cosmetic products.

The present invention preferably provides compositions comprising components of genetically modified plant cells according to the invention, of genetically modified plants according to the invention, of propagation material according to the invention, of harvestable plant parts according to the invention or of plants obtainable by a process according to the invention and comprising recombinant nucleic acid molecules, where the recombinant nucleic acid molecules are characterized in that they comprise nucleic acid molecules coding for a hyaluronan synthase and proteins having the (enzymatic) activity of a GFAT.

A stable integration of foreign nucleic acid molecules into the genome of a plant cell or plant results in the foreign nucleic acid molecules being flanked after integration into the genome of a plant cell or plant by genomic plant nucleic acid sequences. Accordingly, in a preferred embodiment, compositions according to the invention are characterized in that the recombinant nucleic acid molecules present in the composition according to the invention are flanked by genomic plant nucleic acid sequences.
Here, the genomic plant nucleic acid sequences may be any sequences naturally present in the genome of the plant cell or plant used for preparing the composition.

The recombinant nucleic acid molecules present in the compositions according to the invention may be individual or various recombinant nucleic acid molecules which nucleic acid molecules coding for a hyaluronan synthase and proteins having the (enzymatic) activity of a GFAT are present on a nucleic acid molecule, or those where the nucleic acid molecules may be present on separate recombinant nucleic acid molecules. Nucleic acid molecules coding for a hyaluronan synthase or coding for a protein having the (enzymatic) activity of a GFAT may be present together on a single recombinant nucleic acid molecule, or two of the nucleic acid molecules mentioned may be present together on a single recombinant nucleic acid molecule and the third nucleic acid molecule may be present on another recombinant nucleic acid molecule in any possible combination, or all nucleic acid molecules mentioned

may in each case be present on individual separate recombinant nucleic acid molecules. Depending on how the nucleic acid molecules coding for a hyaluronan synthase or coding for a protein having the (enzymatic) activity of a GFAT are present in a composition according to the invention, they may be flanked by identical or different genomic plant nucleic acid sequences.

That compositions according to the invention comprise recombinant nucleic acid molecules may be demonstrated using methods known to the person skilled in the art, such as, for example, methods based on hybridization or, preferably, using methods based on PCR (polymerase chain reaction).

Preferably, compositions according to the invention comprise at least 0.005%, with preference at least 0.01%, particularly preferably at least 0.05% and especially preferably at least 0.1% of hyaluronan.
Preferably, compositions according to the invention comprise at most 5%, with preference at most 2%, particularly preferably at most 1% and especially preferably at least 0,5% of hyaluronan.

As already mentioned above, it is possible to use genetically modified plant cells according to the invention, genetically modified plants according to the invention, propagation material according to the invention, harvestable plant parts according to the invention, processible plant parts according to the invention, consumable plant parts according to the invention or plants obtainable by a process according to the invention to prepare foodstuff or feedstuff. However, use as raw materials for industrial applications is also possible, without hyaluronan having to be isolated. Thus, for example, genetically modified plants according to the invention or parts of genetically modified plants according to the invention can be applied to areas under agricultural cultivation to achieve increased water binding of the soil. Furthermore, genetically modified plants according to the invention or genetically modified plant cells according to the invention can be used for preparing drying agents (for example for use when shipping moisture-sensitive items) or as absorbers of liquids (for example in nappies or for absorbing spilt aqueous liquids). For such applications, it is possible to use entire genetically modified plants according to the invention, parts of

genetically modified plants according to the invention or comminuted (for example ground) genetically modified plants according to the invention or plant parts according to the invention, as required. Suitable for applications in which ground plants or plant parts are used are in particular plant parts containing hyaluronan, but only a low proportion of water. These are preferably grains of cereal plants (corn, rice, wheat, rye, oats, barley, sago or sorghum). Since genetically modified plant cells according to the invention and genetically modified plants according to the invention have a higher hyaluronan content than transgenic plants described in the literature, compared to these less material has to be used for industrial applications when use is made of genetically modified plant cells according to the invention or genetically modified plants according to the invention.

The present invention also provides processes for preparing a composition according to the invention, where genetically modified plant cells according to the invention, genetically modified plants according to the invention, propagation material according to the invention, harvestable plant parts according to the invention, processible plant parts according to the invention, consumable plant parts according to the invention or plants obtainable by a process according to the invention for producing a plant which synthesizes hyaluronan are used. The processes for preparing a composition according to the invention are preferably processes for preparing foodstuff or feedstuff, processes for preparing a pharmaceutical product or processes for preparing a cosmetic product.

Processes for preparing foodstuff or feedstuff are known to the person skilled in the art. Processes for using genetically modified plants according to the invention or plant parts according to the invention in industrial areas are also known to the person skilled in the art and include inter alia comminuting or grinding of genetically modified plants according to the invention or plant parts according to the invention; however, they are not exclusively limited thereto. Some of the advantages resulting from using subject-matters according to the invention for preparing foodstuff/feedstuff or for use in industrial areas have already been described above.

A process according to the invention for preparing a composition is particularly

preferably a process for preparing a composition which comprises hyaluronan.

Compositions obtainable by a process for preparing a composition according to the invention are likewise provided by the present invention.

The present invention also relates to the use of genetically modified plant cells according to the invention, genetically modified plants according to the invention, propagation material according to the invention, harvestable plant parts according to the invention, processible plant parts according to the invention, consumable plant parts according to the invention or plants obtainable by a process according to the invention for producing a plant which synthesizes hyaluronan for preparing a composition according to the invention. Preference is given to the use of genetically modified plant cells according to the invention, genetically modified plants according to the invention, propagation material according to the invention, harvestable plant parts according to the invention, processible plant parts according to the invention, consumable plant parts according to the invention or of plants obtainable by a process according to the invention for producing a plant which synthesizes hyaluronan for preparing foodstuff or feedstuff, for preparing a pharmaceutic or for preparing a cosmetic product.

Description of the sequences

SEQ ID NO 1: Nucleic acid sequence coding for a hyaluronan synthase of *Paramecium bursaria Chlorella* Virus 1.

SEQ ID NO 2: Amino acid sequence of a hyaluronan synthase of the *Paramecium bursaria Chlorella* Virus 1. The amino acid sequence shown can be derived from SEQ ID NO 1.

SEQ ID NO 3: Synthetic nucleic acid sequence coding for a hyaluronan synthase of *Paramecium bursaria Chlorella* Virus 1. The synthesis of the codons of the sequence shown was carried out such that it is adapted to the use of codons in plant cells. The nucleic acid sequence shown codes for a protein having the amino acid

sequence shown under SEQ ID NO 2.

SEQ ID NO 4: Nucleic acid sequence coding for a protein having the activity of a GFAT-1 from the mouse.

SEQ ID NO 5: Amino acid sequence of a protein having the activity of a GFAT-1 from the mouse. The amino acid sequence shown can be derived from SEQ ID NO 4.

SEQ ID NO 6: Nucleic acid sequence coding for a protein having the activity of a GFAT-2 from the mouse.

SEQ ID NO 7: Amino acid sequence of a protein having the activity of a GFAT-2 from the mouse. The amino acid sequence shown can be derived from SEQ ID NO 6.

SEQ ID NO 8: Nucleic acid sequence coding for a protein having the activity of a GFAT from *Escherichia coli*.

SEQ ID NO 9: Amino acid sequence of a protein having the activity of a GFAT from *Escherichia coli*. The amino acid sequence shown can be derived from SEQ ID NO 8.

SEQ ID NO 10: Synthetic nucleic acid sequence coding for a protein having the activity of a GFAT from *Escherichia coli*. The synthesis of the codons of the sequence shown was carried out such that it was adapted to the use of codons in plant cells. The nucleic acid sequence shown codes for a protein having the amino acid sequence shown under SEQ ID NO 9.

SEQ ID NO 11: Synthetic Oligonucleotide used in Example 1.

SEQ ID NO 12: Synthetic Oligonucleotide used in Example 1.

SEQ ID NO 13: Synthetic Oligonucleotide used as PCR primer in Example 10.

SEQ ID NO 14: Synthetic Oligonucleotide used as PCR primer in Example 10.

All literature cited, including but not limited to accession numbers for nucleic acid and amino acid sequences are incorporated into the description by way of reference.

Description of the figures

Fig. 1: Shows a calibration curve and the corresponding equation of the regression line used for calculating the hyaluronan content in plant tissue. The calibration curve was established with the aid of the commercial test kit (Hyaluronic Acid (HA) test kit from Corgenix Inc., Colorado, USA, Prod. No. 029-001) and the standard solutions supplied therewith.

General methods

Methods which can be used in connection with the present invention are described below. These methods are specific embodiments; however, the present invention is not limited to these methods. It is known to the person skilled in the art that the invention can be carried out in the same manner by modifying the methods described and/or by replacing individual methods or parts of methods by alternative methods or alternative parts of methods.

1. Transformation of potato plants

Potato plants were transformed with the aid of *Agrobacterium*, as described in Rocha-Sosa et al. (EMBO J. 8, (1989), 23-29).

2. Isolation of hyaluronan from plant tissue

To detect the presence of hyaluronan and to determine the hyaluronan content in plant tissue, plant material was worked up as follows: 200 µl of water (demineralized, conductivity ≥18 MΩ) were added to about 0.3 g of material, and the mixture was comminuted in a laboratory oscillating ball mill (MM200, from Retsch, Germany, 30 sec at 30 Hz). A further 800 µl of water (demineralized, conductivity ≥18 MΩ) was then added, and the mixture was mixed well (using, for example, a Vortex mixer). Cell debris and insoluble components were separated from the supernatant by centrifuging at 16 000 xg for 5 minutes.

3. Purification of hyaluronan

About 100 grams of tubers were peeled, cut into pieces of a size of about 1 cm^3 and, after addition of 100 ml of water (demineralized, conductivity ≥18 MΩ) comminuted in

a Warring blender at maximum speed for about 30 seconds. The cell debris was then removed using a tea sieve. The cell debris that had been removed was resuspended in 300 ml of water (demineralized, conductivity ≥18 MΩ) and again removed using a tea sieve. The two suspensions obtained (100 ml + 300 ml) were combined and centrifuged at 13 000 xg for 15 minutes. NaCl was added to the centrifugation supernatant obtained until a final concentration of 1% had been reached. After the NaCl had gone into solution, precipitation was carried out by addition of twice the volume of ethanol followed by thorough mixing and incubation at -20°C overnight. The mixture was then centrifuged at 13 000 xg for 15 minutes. The sedimented precipitate obtained after this centrifugation was dissolved in 100 ml of buffer (50 mM TrisHCl, pH 8, 1mM $CaCl_2$) and proteinase K was then added to a final concentration of 100 µg/ml and the solution was incubated at 42°C for 2 hours. This was followed by 10 minutes of incubation at 95°C. Once more, NaCl was added to this solution until a final concentration of 1% had been reached. After the NaCl had gone into solution, another precipitation was carried out by addition of twice the volume of ethanol, thorough mixing and incubation at -20°C for about 96 hours. This was followed by 15 minutes of centrifugation at 13 000 xg. The sedimented precipitate obtained after this centrifugation was dissolved in 30 ml of water (demineralized, conductivity ≥18 MΩ), and once more, NaCl was added to a final concentration of 1%. By adding twice the volume of ethanol, thorough mixing and incubation at -20°C overnight, another precipitation was carried out. The precipitate obtained after subsequent centrifugation at 13 000 xg for 15 minutes was dissolved in 20 ml of water (demineralized, conductivity ≥18 MΩ).

Further purification was carried out by centrifugal filtration. To this end, in each case 5 ml of the dissolved precipitate were applied to a membrane filter (CentriconAmicon, pore width 10 000 NMWL, Prod. No. UCF8 010 96), and the sample was centrifuged at 2200 xg until only about 3 ml of the solution above the filter remained. Two more times, in each case 3 ml of water (demineralized, conductivity ≥18 MΩ) were then added to the solution above the membrane and in each case re-centrifuged under identical conditions until, at the end, only about 3 ml of the solution above the filter remained. The solutions still present above the membrane after centrifugal filtration were taken off, and the membrane was rinsed repeatedly (three to five times) with about 1.5 ml of water (demineralized, conductivity ≥18 MΩ). All solutions which were

still present above the membrane and the solutions obtained from rinsing were combined, NaCl was added to a final concentration of 1%, after the NaCl had gone into solution, twice the volume of ethanol was added, the sample was mixed and a precipitate was obtained by storage at -20°C overnight. The precipitate obtained after subsequent centrifugation at 13 000 xg for 15 minutes was dissolved in 4 ml of water (demineralized, conductivity ≥18 MΩ) and then freeze-dried (24 hours under a pressure of 0.37 mbar, freeze drying apparatus Christ Alpha 1-4 from Christ, Osterode, Germany).

4. Detection of hyaluronan and determination of the hyaluronan content

Hyaluronan was detected using a commercial test (hyaluronic acid (HA) test kit from Corgenix, Inc., Colorado, USA, Prod. No. 029-001) according to the instructions of the manufacturer which are herewith incorporated into the description by way of reference. The test principle is based on the availability of a protein which binds specifically to hyaluronan (HABP) and is carried out similarly to an ELISA, where a color reaction indicates the hyaluronan content in the sample examined. Accordingly, for the quantitative determination of hyaluronan, the samples to be measured should be employed in a concentration such that it is within the stated limits (for example: dilution of the sample in question or use of less water for extracting hyaluronan from plant tissue, depending on whether a limit was exceeded or not reached).

In parallel batches, aliquots of the samples to be determined were initially subjected to hyaluronidase digestion and then measured using the commercial test (hyaluronic acid (HA) test kit from Corgenix, Inc., Colorado, USA, Prod. No. 029-001). Hyaluronidase digestion was carried out using 400 µl of potato tuber extract in hyaluronidase buffer (0.1 M potassium phosphate buffer, pH 5.3; 150 mM NaCl) by adding 5 µg (~3 units) of hyaluronidase (hyaluronidase type III from Sigma, Prod. No. H 2251) and incubating at 37°C for 30 min.

In each case in a dilution of 1:10, all samples were then used for determining the hyaluronan content.

5. Determination of the activity of a GFAT

The activity of a protein having the activity of GFAT is determined as described in Rachel et al. (1996, J. Bacteriol. 178 (8), 2320-2327).

To distinguish whether a protein has the activity of a GFAT-1 or GFAT-2, the method described in Hu et al. (2004, J. Biol. Chem. 279 (29), 29988-29993) is used.

6. Transformation of rice plants

Rice plants were transformed by the method described by Hiei et al. (1994, Plant Journal 6(2), 271-282).

7. Transformation of tomato plants

Tomato plants were transformed with the aid of *Agrobacterium* according to the method described in US 5,565,347.

Examples

1. Preparation of the plant expression vector IR 47-71

The plasmid pBinAR is a derivative of the binary vector plasmid pBin19 (Bevan, 1984, Nucl Acids Res 12: 8711-8721) which was constructed as follows:
A fragment of a length of 529 bp which comprised the nucleotides 6909-7437 of the 35S promoter of the cauliflower mosaic virus was isolated as *EcoR* I/*Kpn* I fragment from the plasmid pDH51 (Pietrzak et al, 1986 Nucleic Acids Res. 14, 5858) and ligated between the *Eco*R I and *Kpn* I restriction sites of the polylinker of pUC18. In this manner, the plasmid pUC18-35S was formed. Using the restriction endonucleases *Hind* III and *Pvu* II, a fragment of a length of 192 bp which included the polyadenylation signal (3′ terminus) of the *Octopin Synthase* gene (gene 3) of the T-DNA of the Ti plasmid pTiACH5 (Gielen et al, 1984, EMBO Journal 3, 835-846) (nucleotides 11 749-11 939) was isolated from the plasmid pAGV40 (Herrera-Estrella et al, 1983 Nature, 303, 209-213). Following addition of *Sph* I linkers to the *Pvu* II restriction site, the fragment was ligated between the *Sph* I and *Hind* III restriction sites of pUC18-35S. This gave the plasmid pA7. Here, the entire polylinker comprising the 35S promoter and *ocs* terminator was removed using *Eco*R I and *Hind* III and ligated into the appropriately cleaved vector pBin19. This gave the plant expression vector pBinAR (Höfgen and Willmitzer, 1990, Plant Science 66, 221-230).

The promoter of the patatin gene B33 from *Solanum tuberosum* (Rocha-Sosa et al.,

1989, EMBO J. 8, 23-29) was, as *Dra* I fragment (nucleotides -1512 - +14), ligated into the *Sst* I-cleaved vector pUC19 whose ends had been blunted using T4-DNA polymerase. This gave the plasmid pUC19-B33. From this plasmid, the B33 promoter was removed using *EcoR* I and *Sma* I and ligated into the appropriately restricted vector pBinAR. This gave the plant expression vector pBinB33.

To facilitate further cloning steps, the MCS (Multiple Cloning Site) was extended. To this end, two complementary oligonucleotides were synthesized, heated at 95°C for 5 minutes, slowly cooled to room temperature to allow good fixation (annealing) and cloned into the *Sal* I and *Kpn* I restriction sites of pBinB33. The oligonucleotides used for this purpose had the following sequence:

5'-TCg ACA ggC CTg gAT CCT TAA TTA AAC TAg TCT CgA ggA gCT Cgg TAC-3'

5'-CgA gCT CCT CgA gAC TAg TTT AAT TAA ggA TCC Agg CCT g-3'

The plasmid obtained was named IR 47-71.

2. Preparation of the plant expression vector pBinARHyg

The fragment comprising the 35S promoter, the *ocs* terminator and the entire Multiple Cloning Site was removed from pA7 using the restriction endonucleases *Eco*R I and *Hind* III and cloned into the vector pBIBHyg (Becker, 1990, Nucleic Acids Res. 18, 203) which had been cut using the same restriction endonucleases. The plasmid obtained was named pBinARHyg.

3. Preparation of the plant expression vector pBinB33-Hyg

The *Eco*RI-*Hind*III fragment comprising the B33 promoter, part of the polylinker and the *ocs* terminator was excised from the plasmid pBinB33 and ligated into the appropriately restricted vector pBIB-Hyg (Becker, 1990, Nucleic Acids Res. 18, 203). The plant expression vector obtained was named pBinB33-Hyg.

4. Synthesis of nucleic acid molecules

a) Synthesis of nucleic acid molecules coding for a hyaluronan synthase of *Paramecium bursaria Chlorella* Virus 1

The nucleic acid sequence coding for a hyaluronan synthase (HAS) of *Paramecium bursaria Chlorella* Virus 1 was synthesized by Medigenomix GmbH (Munich, Germany) and cloned into the vector pCR2.1 from Invitrogen (Prod. No. K2000-01).

The plasmid obtained was named IC 323-215. The synthetic nucleic acid sequence coding for the HAS protein from *Paramecium bursaria Chlorella* Virus 1, is shown under SEQ ID NO 3. The corresponding nucleic acid sequence originally isolated from the *Paramecium bursaria Chlorella* Virus 1 is shown under SEQ ID NO 1.

b) Synthesis of nucleic acid molecules coding for a protein having the activity of a GFAT from *Escherichia coli*

The nucleic acid sequence coding for a protein having the activity of a GFAT from *Escherichia coli* was synthesized by Entelechon GmbH and cloned into the vector pCR4Topo from Invitrogen (Prod. No. K4510-20). The plasmid obtained was named IC 373-256. The synthetic nucleic acid sequence coding for a protein having the activity of a GFAT from *Escherichia coli*, is shown under SEQ ID NO 10. The corresponding nucleic acid sequence originally isolated from *Escherichia coli* is shown under SEQ ID NO 8.

5. Origin of further nucleic acid molecules

a) Nucleic acid molecules coding for a protein having the activity of a GFAT-1 from the mouse

The nucleic acid sequence coding for a protein having the activity of a GFAT-1 was purchased from BioCat GmbH, Heidelberg (Art.No. MMM1013-65346, cDNA clone MGC:58262, IMAGE:6742987). This is a clone produced by I.M.A.G.E. Konsortium (http://image.llnl.gov) and distributed by BioCat GmbH. Here, the cDNA coding for a protein having the activity of a GFAT-1 was cloned into the vector pCMV Sport 6 from Invitrogen. The plasmid obtained was named IC 365-256. The nucleic acid sequence, inserted into IC 365-256, coding for a protein having the activity of a GFAT-1 from *Mus musculus* has, compared to the nucleic acid sequence shown under SEQ ID NO 4, a base exchange from T to C in position 1090 and a base exchange from G to A in position 2027. These base exchanges do not result in amino acid exchanges of the amino acid sequences coded for by the two different nucleic acid molecules.

The coding nucleic acid sequence for the protein having the activity of a GFAT-1 from the mouse is shown in SEQ ID NO 4.

To facilitate subsequent cloning steps, the sequence coding for a protein having the

activity of a GFAT-1 was isolated using the restriction endonucleases *Xho* I and *Eco* RV from IC 365-256 and cloned into the plasmid pME9 (pBlueSkript vector from Stratagene) having a modified multiple cloning site which additionally has a *Pac* I restriction site at both ends, which plasmid had been cut with the same restriction endonucleases. The plasmid obtained was named IC 367-256.

b) Nucleic acid molecules coding for a protein having the activity of a GFAT-2 from the mouse

Nucleic acid molecules coding for a protein having the activity of a GFAT-2 from the mouse were purchased from Invitrogen (Clone ID 4167189, cDNA clone MGC:18324, IMAGE:4167189). This is a clone which is produced by I.M.A.G.E. Konsortium (http://image.llnl.gov) and distributed by Invitrogen. Here, the cDNA coding for a protein having the activity of a GFAT-2 is cloned into the vector pCMV Sport 6 from Invitrogen. The plasmid was named IC 369-256. The nucleic acid sequence coding for the protein having the activity of a GFAT-2 from *Mus musculus* is shown under SEQ ID NO 6.

6. Preparation of the plant expression vector IC 341-222 which comprises a coding nucleic acid sequence for a hyaluronan synthase of *Paramecium bursaria* Chlorella virus 1

Using restriction digestion with *BamH* I and *Xho* I, nucleic acid molecules comprising the coding sequence of hyaluronan synthase were isolated from the plasmid IC 323-215 and cloned into the *BamH* I and *Xho* I restriction sites of the plasmid IR 47-71. The plant expression vector obtained was named IC 341-222.

7. Preparation of the plant expression vector IC 399-299 comprising a coding nucleic acid sequence for a protein having the activity of a GFAT-2 from the mouse

Using restriction digestion with *Xho* I and *Asp* 718, nucleic acid molecules comprising the coding sequence for a protein having the activity of a GFAT-2 from the mouse were isolated from the plasmid 369-256 and cloned into the plant expression vector pBinB33-Hyg which had been cut with the same restriction endonucleases. The plant expression vector obtained was named IC 399-299.

8. Preparation of the plant expression vector IC 399-300 comprising a coding nucleic acid sequence for a protein having the activity of a GFAT from *E. coli*

Nucleic acid molecules comprising the coding sequence of the protein having the activity of a GFAT from *E. coli* were isolated from the plasmid 373-256 by restriction digestion with *Sac* I and *Sbf* I and cloned into the plant expression vector pBinB33-Hyg which had been cut with the same restriction endonucleases. The plant expression vector obtained was named IC 399-300.

9. Preparation of the plant expression vector pBA16, which contains a coding nucleic acid sequence for a hyaluronan synthase of *Paramecium bursaria Chlorella* virus 1

Using the restriction endonuclease *Asp* 718I, a fragment comprising the coding nucleic acid sequence for a hyaluronan synthase of *Paramecium bursaria Chlorella* virus 1 was isolated from the plasmid IC 323-215, the ends of the fragment were blunted using Klenow polymerase and the resulting fragment was then once more cleaved using the restriction endonuclease *Pac* I. The fragment obtained in this manner was ligated into the plasmid IR103-123 (described in WO 2006 032538), which had been cleaved using the restriction endonucleases *Pac* I and *Ecl136* II. The plant expression vector obtained was referred to as pBA16.

10. Preparation of the plant expression vector IC 386-299

The DNA of the prolamin promoter from rice (EMBL Accession NO D63901, Sha *et al.*, 1996, Biosci. Biotech. Biochem. 60, 335 – 337, Wu *et al.*, 1998. Plant Cell Physiol. 39(8), 885 – 889) was amplified by using genomic DNA isolated from leaves of *Oryza sativa* (cultivar M202).

Conditions used for PCR amplification:

For amplification, the DNA polymerase Expand High Fidelity (PCR Systems, Roche Prod. Nr.: 1732641) was used The conditions and buffers supplied by the manufacturer of the afore mentioned kit were used.

DNA: 50 ng of genomic rice DNA

dNTPs: 0,83 µM dNTP Mix

0,25 µM Pimer prol-F1
5'-AAAAACTAGTTCTACATCGGCTTAGGTGTAGCAACACG
0.25 µM Primer prol-R1
5'- AAAAGATATCTGTTGTTGGATTCTACTACTATGCTTCAA

Reaction cnditions: Step 1 94°C 15 sec
Step 2 60°C 15 sec
Step 3 72°C 45 sec

Steps 1 to 3 were repeated 35 times before the reaction was cooled to 4°C.

The fragment obtained by PCR amplification was cloned by the use of the TA cloning kit (Invitrogen Prod. Nr.: KNM2040-01) into the plasmid pCR 2.1. The resulting plasmid was named MI 4-154.

A nucleic acid fragment comprising the coding sequence of the protein having the activity of a GFAT-2 from the mouse was isolated from plasmid IC 369-256 by using the resetriction endonucleases *Not* I und *Kpn* I and cloned into the *Not* I und *Kpn* I sites of the vector pMCS5 (purchased from MoBiTec). The plasmid obtained was named IC 385-299. The nucleic acid fragment omprising the coding sequence of the protein having the activity of a GFAT-2 from the mouse was isolated from plasmid IC 385-299 by using the resetriction endonucleases *Xho* I und *Hpa* I and cloned into the *Xho* I and *Ecl* 136 II restriction sites of plasmid MI 9-154. The obtained plant expression vector was named IC 386-299.

Basis for the preparation of vector MI 9-154 was the plasmid ML 18-56 (WO 05 030941). A multiple cloning site (MCS) comprising sticky ends for cloning into the restriction sites *Hind* III and *Pst* I and comprising the additional restriction sites *Pst* I, *Sac* I, *Bln* I, *Xho* I, *Hpa* I, *Spe* I and *Hind* III was prepared by annealing of two complementary synthetic oligonucleotides. The annealed oligonucleotide was cloned into the restriction sites *Hind* III and *Pst* I of plasmid ML 18-56. The vector obtained was named MI 8-154. The nucleic acid fragment comprising the prolamin promoter was isolated from plasmid MI 4-154 by using the restriction endonucleases *Eco* RV und *Spe* I and cloned into the vector MI 8-154. The obtained plasmid was named MI 9-154.

11. Transformation of plants with plant expression vectors comprising nucleic acid molecules coding for a hyaluronan synthase

Potato plants (cv Désirée) were transformed using the plant expression vector IC 341-222, which comprises a coding nucleic acid sequence for a hyaluronan synthase from *Paramecium bursaria Chlorella* virus 1 under the control of the promoter of the patatin gene B33 from *Solanum tuberosum* (Rocha-Sosa et al., 1989, EMBO J. 8, 23-29) using the method given under General Methods item 1. The transgenic potato plants obtained, which were transformed with the plasmid IC 341-222, were named 365 ES.

12. Analysis of the transgenic plants transformed with plant expression vectors comprising nucleic acid molecules coding for a hyaluronan synthase

a) Construction of a calibration curve

A calibration curve was constructed using the standard solutions supplied with the commercial test kit (hyaluronic acid (HA) test kit from Corgenix, Inc., Colorado, USA, Prod. No. 029-001), according to the methods described by the manufacturer. To determine the extinction at 1600 ng/ml of hyaluronan, double the amount, based on the amount of supplied standard indicated by the manufacturer, comprising 800 ng/ml of hyaluronan was used. In each case, three independent measurement series were carried out, and the corresponding mean was determined. This gave the following calibration curve:

Hyaluronan concentration	Independent individual measurements			Mean	n.d.
	E_{450nm}	E_{450nm}	E_{450nm}		
0 ng/ml	0.100	0.096	0.096	0.097	0.002
50 ng/ml	0.224	0.183	0.222	0.210	0.023
100 ng/ml	0.396	0.263	0.377	0.345	0.072
200 ng/ml	0.554	0.443	0.653	0.550	0.105
500 ng/ml	1.231	0.850	1.221	1.101	0.217
800 ng/ml	1.465	1.265	1.795	1.508	0.268
1600 ng/ml	2.089	2.487	3.170	2.582	0.547

Table 1: Values for constructing a calibration curve for the quantitative determination of the hyaluronan content in plant tissue. With the aid of software (Microsoft Office Excel 2002, SP2), the measured values obtained were entered into a diagram and the equation of the function of the trend line was determined (see Fig 1). E_{450nm} refers to the extinction at a wavelength of 450 nm, s.d. is the standard deviation of the calculated mean of the individual values.

b) Analysis of potato tubers of lines 365 ES

In a greenhouse, individual plants of the line 365 ES were cultivated in soil in 6 cm pots. In each case about 0.3 g of material of potato tubers of the individual plants was processed according to the method described under General Methods item 2. Using the method described under General Methods item 4, the amount of hyaluronan present in the respective plant extracts was determined, with the aid of the calibration curve shown in Example 12a) and Fig. 1. Here, the supernatant obtained after centrifugation was used in a dilution of 1:10 for determining the hyaluronan content. For selected plants, the following results were obtained:

Name of the plant	Weight of the plant material employed [g]	Extinction E450	Amount of hyaluronan [ng/ml]	Hyaluronan based on the fresh weight of the plant material [µg/g]
365 ES 13	0.297	2.746	14	47
365 ES 74	0.306	4.000	20816	68
Wild-type	0.305	0.111	n.d.	n.d.

Table 2: Amount of hyaluronan (in µg of hyaluronan per g of fresh weight) produced by independent transgenic plants of the line 365 ES. Column 1 refers to the plant from which tuber material was harvested (here, "wild-type" refers to untransformed plants which, however, have the genotype used as starting material for the transformation). Column 2 indicates the amount of tuber material of the plant in question used for determining the hyaluronan content. Column 3 contains the measured extinction of a 1:10 dilution of the respective plant extract. Column 4 was calculated with the aid of the regression line equation (see Fig. 1) taking into account the dilution factor, as follows: ((value column 3 - 0.149)/0.00185) x 10. Column 5 indicates the amount of hyaluronan based on the fresh weight used and was calculated as follows: (value column 4/value column 2)/1000. "n.d." means not detectable.

13. Transformation of hyaluronan-synthesizing plants with plant expression vectors comprising coding nucleic acid sequences for a protein having the activity of a GFAT from *Escherichia coli*

a) Transformation of plants

Potato plants of the lines 365 ES 13 and 365 ES 74 were in each case transformed with the plant expression vector IC 399-300 using the method given under General Methods item 1. The transgenic potato plants obtained after transformation of line 365 ES 74 with the plasmid IC 399-300, were named 433 ES.

b) Analysis of potato tubers of line 433 ES

In a greenhouse, individual plants of the line 433 ES were cultivated in soil in 6 cm pots. In each case about 0.3 g of material of potato tubers and/or leaves of the individual plants was processed according to the method described under General Methods item 2. Using the method described under General Methods item 4, the amount of hyaluronan present in the respective plant extracts was determined, with the aid of the calibration curve shown in Example 12a) and Fig. 1. Here, the supernatant obtained after centrifugation was used in a dilution of 1:10 for determining the hyaluronan content. For selected plants, the following results were obtained:

Name of the plant	Amount of HA in leaves [µg/g FG]	Amount of HA in tubers [µg/g FG]
433ES 1	111,84	126,70
433ES 3	303,34	203,16
433ES 4	3142,41	
433ES 5	312,98	825,96
433ES 7	1492,94	
433ES 8	914,03	
433ES 9	1858,68	
433ES 10	357,90	
433ES 11	5962,82	
433ES 12		662,99
433ES 13	626,52	624,33
433ES 14	665,23	
433ES 15	601,36	
433ES 16	3416,94	
433ES 18	781,02	
433ES 19	3294,09	
433ES 20	1348,85	975,18
433ES 21	937,92	
433ES 22	1086,45	
433ES 23	1327,28	
433ES 24	340,80	76,00
433ES 25	1529,95	
433ES 26	375,53	
433ES 27		425,65

Name of the plant	Amount of HA in leaves [µg/g FG]	Amount of HA in tubers [µg/g FG]
433ES 28	1850,99	294,98
433ES 30	2512,40	
433ES 31	3337,54	
433ES 32	1583,60	
433ES 34	3552,44	
433ES 35	5419,43	
433ES 36	902,01	
433ES 37	829,35	
433ES 38	1536,55	
wt-1	0,40	n.d.
wt-2	0,34	n.d.
wt-3	n.d.	
365 ES 74-1	265,1	
365 ES 74-2		91,84
365 ES 74-3	193,5	
365 ES 74-4		175,48
365 ES 74-5	73,9	
365 ES 74-6		168,68
365 ES 74-7	67,58	
365 ES 74-8		121,89
365 ES 74-9	62,23	
365 ES 74-10		275,24
365 ES 74-11	134,56	

Tabelle 3: Amount of hyaluronan („HA" in µg of hyaluronan per g of fresh weight) produced by independent transgenic plants of the line 433 ES. Column 1 refers to the plant from which tuber or leaf material was harvested (here, "wild-type" refers to

untransformed plants and ES 365 74 refers to plants which have been used as starting material for transformation with plasmid C 399-300). Columns 2 and 3 refer to the amount of hyaluronan detected in leaves or tubers, respectively.

14. Transformation of rice plants

a) With plant expression vectors comprising nucleic acid molecules coding for a hyaluronan synthase from *Paramecium bursaria Chlorella* virus 1

Rice plants (cultivar M202) were transformed with the plant expression vector pBA16, which contains a coding nucleic acid sequence for a hyaluronan synthase protein from *Paramecium bursaria Chlorella* virus 1 under the control of the promoter of the globulin gene from *Oryza sativa* (Wu et al., 1998, Plant Cell Physiol. 39(8), 885-889), using the method given under General Methods item 6. The transgenic rice plants obtained which had been transformed with the plasmid pBA16 were referred to as Os-pBA16.

b) With plant expression vectors comprising coding nucleic acid sequences for a protein having the activity of a GFAT-2 from mouse

Rice plants (cultivar M202) were transformed with the plant expression vector IC 386-299, which contains a coding nucleic acid sequence for a protein having the activity of a GFAT-2 from the mouse under the control of the promoter of the 13-kDa prolamin polypeptids from *Oryza sativa*, using the method given under General Methods item 6. The transgenic rice plants obtained which had been transformed with the plasmid pBA16 were referred to as GAOS0788.

15. Analysis of rice plants of the line Os-pBA16

a) Immature rice seeds

Immature rice seeds (5 to 10 days after pollination) produced by individual plants of the line OS-pBA16, cultivated in soil in the greenhouse were collected, frozen in liquid nitrogen and stored at -80 °C. Three frozen grains of each individual plant were selected randomly, the endosperm was squeezed out, pooled, weighted, and frozen in liquid nitrogen again. The sample was broken up with a Ball mill (Modell MM200, Firma Retsch, Germany), 100 µl Water was added, the homogenate was mixed, centrifuged (13000xg, 5 min) and the hyaluronan concentration of each sample was determined according to the method described under General Methods, item 4.

Out of 37 seed pools, each comprising 3 immature seeds from independent plants of line OS-pBA16 more than 70% proved to synthesize a significant amount of hyaluronan (at least 0,1 µg hyaluronan per g fresh weight) in seeds. The amount of hyaluronan in seed pools prepared from independent rice plants varied between 0,1 and 15,7 µg hyaluronan per g fresh weight. Results for seed pools each prepared from independent plants are shown in the following table:

Plant material	Hyaluronan based on the fresh weight of the plant material [µg/g]
OS-pBA16 0612-00102	7,30
OS-pBA16 0612-00102	0,54
OS-pBA16 0612-00201	12,16
OS-pBA16 0612-00401	1,12
OS-pBA16 0612-00402	7,28
OS-pBA16 0612-00502	0,08
OS-pBA16 0612-00601	0,37
OS-pBA16 0612-00701	0,66
OS-pBA16 0612-00702	0,03
OS-pBA16 0612-00801	2,48
OS-pBA16 0612-00802	3,84
OS-pBA16 0612-00902	0,02
OS-pBA16 0612-01001	0,02
OS-pBA16 0612-01201	1,71
OS-pBA16 0612-01202	0,11
OS-pBA16 0612-01301	5,84
OS-pBA16 0612-01401	0,25
OS-pBA16 0612-01402	0,11
OS-pBA16 0612-01501	0,16
OS-pBA16 0612-01601	1,12
Wild type-1	0,01
Wild type-2	0,02
Wild type-3	0,02
OS-pBA16 0613-00101	4,43
OS-pBA16 0613-00102	1,95
OS-pBA16 0613-00301	0,25
OS-pBA16 0613-00401	15,72
OS-pBA16 0613-00402	0,38
OS-pBA16 0613-00502	0,87
OS-pBA16 0613-00601	0,02
OS-pBA16 0613-00602	0,01

Plant material	Hyaluronan based on the fresh weight of the plant material [µg/g]
OS-pBA16 0613-00701	0,23
OS-pBA16 0613-00702	0,80
OS-pBA16 0613-00801	1,72
OS-pBA16 0613-00802	0,15
OS-pBA16 0613-00902	0,02
OS-pBA16 0613-01001	0,02
OS-pBA16 0613-01002	0,01
OS-pBA16 0613-01102	0,24
OS-pBA16 0613-01202	9,48
OS-pBA16 0613-01301	13,44
OS-pBA16 0613-01302	9,79
OS-pBA16 0613-01501	0,63
OS-pBA16 0613-01502	6,78

Table 4: Detection of hyaluronan in seed pools, each prepared from independent plants of the transgenic line OS-pBA16.

b) Rice flour

20-25 mature seeds were harvested from each transformed plant. Husks were removed by a dehusker (Laboratory Paddy sheller, Grainman, Miami, Florida, USA) and brown rice grain was milled with a laboratory mill (Cyclotec, Sample mill, Foss, Denmark). To about 40 mg of the obtained rice flour from the pooled seeds of each independent plant, 1 ml water was added, the sample was mixed, centrifuged (13000xg, 5 min) and the hyaluronan concentration of the supernatant of each sample was determined according to the method described under General Methods, item 4. Results for selected flour samples prepared from independent plants are shown in the following table:

Plant material	Hyaluronan based on the weight of the plant material [µg/g]
OS-pBA16 0612-00101	2,03
OS-pBA16 0612-00102	1,19
OS-pBA16 0612-00201	1,94
OS-pBA16 0612-00402	4,24
OS-pBA16 0612-00502	1,19
OS-pBA16 0612-00601	1,64
OS-pBA16 0612-00602	2,51
OS-pBA16 0612-00701	0,87
OS-pBA16 0612-00702	1,04
OS-pBA16 0612-00801	3,61
OS-pBA16 0612-00802	3,88
OS-pBA16 0612-00902	1,02
OS-pBA16 0612-01001	0,58
OS-pBA16 0612-01201	4,86
OS-pBA16 0612-01202	2,96
OS-pBA16 0612-01301	11,30
OS-pBA16 0612-01401	1,64
OS-pBA16 0612-01402	1,50
OS-pBA16 0612-01501	4,54
OS-pBA16 0612-01601	1,90
OS-pBA16 0613-00101	3,46
OS-pBA16 0613-00102	3,94
OS-pBA16 0613-00301	3,32
OS-pBA16 0613-00401	5,21
OS-pBA16 0613-00402	3,45
OS-pBA16 0613-00502	5,20
OS-pBA16 0613-00601	0,83
OS-pBA16 0613-00602	0,77
OS-pBA16 0613-00701	2,63
OS-pBA16 0613-00702	3,77
OS-pBA16 0613-00801	1,55
OS-pBA16 0613-00802	2,81

Plant material	Hyaluronan based on the weight of the plant material [µg/g]
OS-pBA16 0613-00902	2,65
OS-pBA16 0613-01001	1,06
OS-pBA16 0613-01002	0,59
OS-pBA16 0613-01102	1,19
OS-pBA16 0613-01202	10,18
OS-pBA16 0613-01301	5,02
OS-pBA16 0613-01302	3,84
OS-pBA16 0613-01501	4,00
OS-pBA16 0613-01502	5,63
OS-pBA16 0613-000101	0,63
OS-pBA16 0613-000103	0,58
OS-pBA16 0613-000104	0,87

Table 5: Detection of hyaluronan in rice flour samples, prepared from seeds of each independent plant of the transgenic line OS-pBA16. Detection was carried out using the method described under General Methods item 4.

b) Analysis of rice plants of the line GAOS0788

Independent rice plants of the line GAOS0788 obtained after transformation with the plasmid IC 386-299 wre cultivated in soil in the greenhouse. From each plant 20-25 mature seeds (grains) were harvested, husks were removed by a dehusker (Laboratory Paddy sheller, Grainman, Miami, Florida, USA) and ca. 7 brown rice grains from each line were milled with a laboratory ball mill (MM200, Company Retsch, Germany, 30 sec. bei 30 HZ), leading to rice flour. Afterwards the content of N-acetylated glucosamin derivatives in each sample was dertemined according to the method as described by Elson and Morgan (1933, J Biochem. 27,1824). Several samples analysed did show an increase in the content of N-acetylated glucosamin derivatives ranging from ca. 2 µmol up to ca. 20 µmol N-acetylated glucosamin derivatives per gram fresh weight of the sample. Single grains of selected plants (GAOS0788-00501), analysed as described above, did show a content of N-acetylated glucosamin derivatives up to ca. 43 µmol per gram fresh weight of the sample.

Selected plants of the lines OS-pBA16 and GAOS0788 will be crossed with each other to obtain plants comprising nucleic acid molecules coding for a protein of a hyaluronan synthase and coding for a protein having the activity of a GFAT-2.

5

Claims

1. A genetically modified plant cell which has a nucleic acid molecule coding for a hyaluronan synthase stably integrated into its genome, wherein said plant cell additionally has an increased activity of a protein having the activity of a glutamine:fructose 6-phosphate amidotransferase (GFAT) compared to corresponding not genetically modified wild-type plant cells.

2. The genetically modified plant cell as claimed in claim 1, wherein the increased activity of a protein having the activity of a glutamine:fructose 6-phosphate amidotransferase (GFAT) is caused by introduction of a foreign nucleic acid molecule into the plant cell.

3. The genetically modified plant cell as claimed in claim 2, wherein the foreign nucleic acid molecule codes for a protein having the enzymatic activity of a glutamine:fructose 6-phosphate amidotransferase (GFAT).

4. The genetically modified plant cell as claimed in any of claims 1, 2 or 3 which synthesizes an increased amount of hyaluronan compared to plant cells having the activity of a hyaluronan synthase and no increased activity of a glutamine:fructose 6-phosphate amidotransferase (GFAT).

5. A plant comprising genetically modified plant cells as claimed in any of claims 1 to 4.

6. Propagation material of plants as claimed in claim 5, comprising genetically modified plant cells as claimed in any of claims 1 to 4.

7. Harvestable plant parts of plants as claimed in claim 5, comprising genetically modified plant cells as claimed in any of claims 1 to 4.

8. A process for producing a plant which synthesizes hyaluronan, which comprises

   a) genetically modifying a plant cell, where the genetic modification comprises steps i to ii below

i) introduction of a foreign nucleic acid molecule encoding for a hyaluronan synthase into the plant cell

ii) introduction of a genetic modification into the plant cell, the genetic modification resulting in an increase of the activity of a protein having the enzymatic activity of a glutamine:fructose 6-phosphate amidotransferase (GFAT) compared to corresponding not genetically modified wild-type plant cells

where steps i to ii can be carried out in any order, individually, or any combinations of steps i to ii can be carried out simultaneously

b) regenerating a plant from plant cells from step a);

c) generating, if appropriate, further plants using the plants according to step b), where, if appropriate, plant cells are isolated from plants obtained according to steps b) i or b) ii and the process steps a) to c) are repeated until a plant is generated which has a foreign nucleic acid molecule coding for a hyaluronan synthase and has an increased activity of a protein having the enzymatic activity of a GFAT compared to corresponding not genetically modified wild-type plant cells.

9. A process for preparing hyaluronan which comprises the step of extracting hyaluronan from genetically modified plant cells as claimed in any of claims 1 to 4, from plants as claimed in claim 5, from propagation material as claimed in claim 6, from harvestable plant parts as claimed in claim 7 or from plants obtainable by a process as claimed in claim 8.

10. Use of a genetically modified plant cell as claimed in any of claims 1 to 4, a plant as claimed in claim 5, propagation material as claimed in claim 6, harvestable plant parts as claimed in claim 7 or of plants obtainable by a process as claimed in claim 8 for preparing hyaluronan.

11. A composition comprising genetically modified plant cells as claimed in any of claims 1 to 4.

12. A process for preparing a composition comprising hyaluronan where genetically modified plant cells as claimed in any of claims 1 to 4, plants as claimed in claim 5, propagation material as claimed in claim 6, harvestable plant parts as claimed in claim 7 or plants obtainable by a process as claimed in claim 8 are used.

13. The use of genetically modified plant cells as claimed in any of claims 1 to 4, of plants as claimed in claim 5, of propagation material as claimed in claim 6, of harvestable plant parts as claimed in claim 7 or of plants obtainable by a process as claimed in claim 8 for preparing a composition as claimed in claim 11.

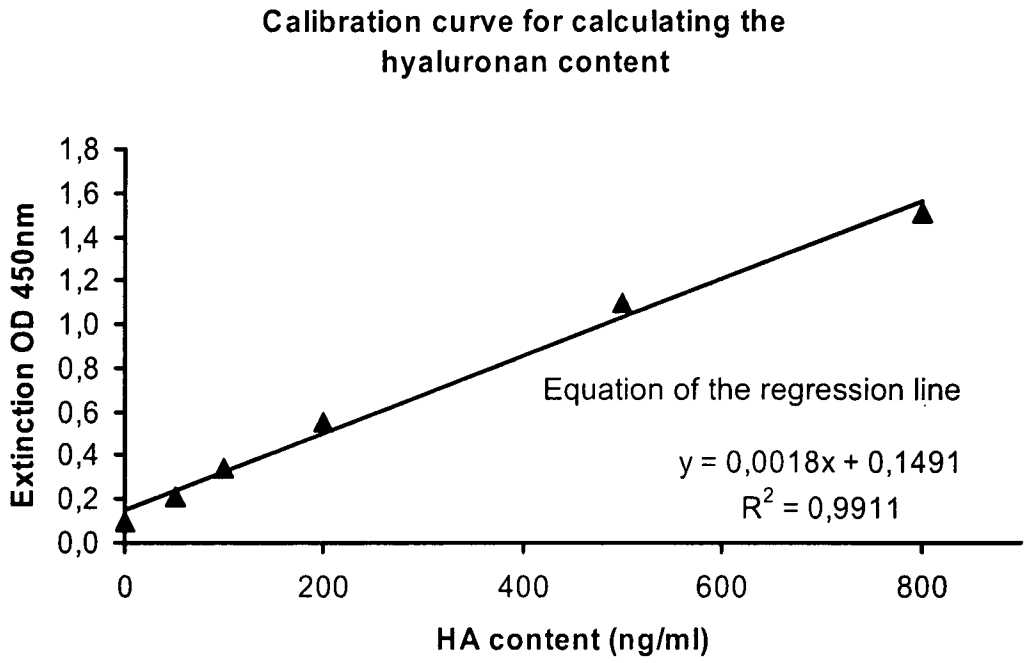

Calibration curve for calculating the hyaluronan content
Extinction OD 450nm
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
200
400
600
800
HA content (ng/ml)
Equation of the regression line
y = 0,0018x + 0,1491
R2 = 0,9911

Fig. 1

PCT/EP2006/009773

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

DSMZ
Deutsche Sammlung von
Mikroorganismen und
Zellkulturen GmbH

INTERNATIONAL FORM

Bayer CropScience GmbH

Brüningstr. 50

65929 Frankfurt/Main

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
IC341-222	DSM 16664

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

(x) a scientific description
( ) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2004-08-25 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit)
and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name:	DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH
Address:	Mascheroder Weg 1b D-38124 Braunschweig

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

U. Wahls

Date: 2004-08-31

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page) 12/2001

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

DSMZ
Deutsche Sammlung von
Mikroorganismen und
Zellkulturen GmbH

INTERNATIONAL FORM

Bayer CropScience GmbH

Brüningstr. 50

65929 Frankfurt/Main

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Bayer CropScience GmbH Brüningstr. 50 Address: 65929 Frankfurt/Main	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 16664 Date of the deposit or the transfer[1]: 2004-08-25

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2004-08-25
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): V. Wells Date: 2004-08-31

1 Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
2 In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
3 Mark with a cross the applicable box.
4 Fill in if the information has been requested and if the results of the test were negative.