Title: TRANSGENIC PLANTS EXPRESSING COBALAMIN BINDING PROTEINS

Abstract: The present invention relates to the use of transgenic plants for the expression of vitamin B12 (cobalamin) binding proteins. Plant cells are transformed with nucleotide sequences adapted for expression and secretion of vitamin B12 binding proteins. The present invention also relates to the use of recombinant vitamin B12 binding proteins from plants in analytical tests and treatment of vitamin B12 deficiency. Also disclosed is a method for purification of recombinant vitamin B12 binding proteins.
TRANSGENIC PLANTS EXPRESSING COBALAMIN BINDING PROTEINS

The present invention generally relates to transgenic plant production of recombinant proteins which are able to bind cobalamin. The recombinant proteins may be used 1) prophylactically and/or therapeutically to treat cobalamin deficiency and 2) analytically.

Two groups of proteins are characterized by specific binding to vitamin B12, (cobalamin), hereafter called cobalamin binding proteins (CBP). One group consists of proteins involved in the assimilation of cobalamin and the other group is involved in the metabolism and utilization of cobalamin and includes the enzymes using cobalamin as a cofactor. Human assimilation of dietary cobalamin (Cbl) is a complex process with three successive Cbl-transporters involved: haptocorrin (HC), intrinsic factor (IF) and transcobalamin (TC) (1-4). These proteins strongly bind to Cbl which is synthesized by bacteria. The three CBPs provide uptake of the Cbl from the food to the body. IF is the main binder of Cbl in the intestinal tract and the IF-Cbl complex binds to an intestinal receptor resulting in the internalization of the Cbl to the blood (3, 4). In the blood the TC-Cbl complex distributes assimilated Cbl among the tissues where it binds to one or more specific receptors present in the cell membrane (2, 4). A significant amount of Cbl circulates in blood bound to HC (1, 4). The exact function of HC is not identified. Inherited HC deficiency does not provoke any visible pathological effect (5) in contrast to the cases of IF- and TC-deficiencies (6, 7).

Two Cbl dependent enzymatic reactions have been identified in mammals. The conversion of methylmalonyl-CoA to succinyl-CoA involves adenosyl-Cbl (Ado-Cbl) as a cofactor. Methyl-Cbl is a cofactor in the synthesis of methionine from homocysteine.
Prior to the present invention, only small amounts of CBP have been isolated. Purification of Cbl binders is complicated by low concentration of these proteins in natural sources (1-4). Isolation of 1 mg of TC requires, for instance, 150-300 liters of human plasma (8, 9). Human IF can be isolated from stomach juice which contain about 1 mg IF per liter. Isolation of pure TC and IF from natural sources is also complicated by the presence of relatively large amounts of HC in these sources. An expression system has been established for IF and TC in insect cells (10, 11) and recombinant proteins were obtained at the level of 10-100 μg. We have expressed human and bovine TC in yeast and obtained 5-7 mg recombinant protein from a total of 1 liter of fermentation media (12). Human IF has been expressed in yeast and the yield was in the range of 1-4 mg per liter of total fermentation media (13). These expression systems are expensive to use and they deliver a mixture of holo- and apo-forms of the CBP. This is also the case for natural sources. The holo-form is the CBP in complex with Cbl, whereas the apo form is a CBP not complexed to Cbl. For use in diagnostic kits and other analytical purposes it is important to achieve purely either the apo- or holo-form of CBP.

IF isolated from human gastric juice is used for diagnostic purposes in the Schilling test where the patient ingests the isolated IF. Therefore, there is a risk for transmission of human diseases from the donor to the patient. Similarly, the use of IF isolated from pig or recombinant human IF isolated from the yeast expression media which contains an enzymatic hydrolysate of meat, may cause a risk for transmission of animal diseases to the patient. The use of recombinant human IF from a plant expression system will eliminate these problems.

Plants do not contain Cbl or CBPs. Therefore, recombinant plants with an inserted gene for i.e. human IF will express only the apo-form of IF. This makes plants very suitable as a "factory" for production of the apo-form of CBPs with no contamination
of the holo-form. In other eukaryotic expression systems the recombinant CBP will be more or less saturated with Cbl resulting in a mixture of holo- and apo-form CBP. All other eukaryotic organisms except plants use Cbl in enzymatic reactions and therefore contain Cbl. Similarly, the use of transgenic plants results in recombinant CBP free of other CBPs since plants do not naturally express CBP. Isolation of IF from other sources, i.e. gastric juice results in a preparation contaminated with some HC since both CBPs are present in gastric juice.

CBPs expressed in plants but not other organisms are only in the apo-form and therefore the plant expression system gives the opportunity for purification of the CBP by affinity column chromatography with a cobalamin column. The holo-form of CBP does not bind to the cobalamin column. Therefore, CBPs expressed in plants are easier to purify than CBPs expressed in other organisms.

CBPs expressed in plants can be designed to have or alternatively not to have identical amino acid sequences as their corresponding native proteins. Posttranslational modifications such as disulphide bond formation between cystein residues will be present in the recombinant proteins. For CBPs with amino acid sequences identical with native CBPs the localization of disulphide bonds are expected to be identical, since CBPs from plants binds Cbl. Therefore the tertiary structure of recombinant CBPs are expected to be identical to native CBPs. The specific binding of recombinant human IF (rhIF) from plants in complex with Cbl to the intestinal receptor cubilin support an identical tertiary structure between native hIF and rhIF from plant. The glycosylation of recombinant CBPs from plants will not be identical to the glycosylation of the corresponding native CPBs, since glycosylation is cell type specific. Therefore, rhIF from plants differ from native IF by the composition of the sugars in the carbohydrate chains.
The plant expression system will be very useful for large scale production of CBP since the growth of recombinant plants is low-tech and furthermore, it is simple to scale up the production of recombinant protein by simply planting more land. The plant expression system gives the opportunity to use large amounts of recombinant CBP in therapeutic programs, e.g. elderly people with reduced uptake of vitamin B12. Furthermore, the CBP may be used after no or very little purification since many plant species, useful for transgenic expression of proteins, are used for human consumption e.g. maize, potato, barley, rice, carrot.

A need exists for a better way to produce these CBPs.

Thus the present invention provides a transgenic plant that expresses at least one protein capable of binding cobalamin and/or analogs thereof. A “cobalamin” (Cbl) is a molecule that consists of a corrin ring with four pyrrole units which surround and bind to the essential and central cobalt atom. Below the corrin plane is a nucleotide derivative with a dimethylbenzimidazole base, which also is linked to the cobalt atom. Finally the cobalt atom binds to a sixth molecule (e.g.:-CH3, OH,-H2O, 5’-deoxyadenosyl, CN) located above the corrin plane. Cyanocobalamin is referred to as vitamin B12, but other cobalamins have identical nutritional properties. Cobalamin cannot be synthesized by animals or plants and is only produced by some microorganisms, in particular, anaerobic bacteria. A “cobalamin analog” is a cobalamin molecule where, for example, the nucleotide unit has been changed or partially removed as in cobinamid. The cobalamin analog may also be a cobalamin linked to another functional molecule, eg a cytotoxin. A “cobalamin binding protein” is a protein which can bind a cobalamin, e.g. the human proteins: intrinsic factor, transcobalamin, haptocorrin, methylmalonyl-CoA mutase and methionine synthetase. A “transgenic
plant” is a plant where some or all of its cells contain an expression vector or a fragment of an expression vector.

In a first preferred embodiment the protein capable of binding cobalamin or analogs thereof would be any one of transcobalamin, intrinsic factor, haptocorrin, methylmalonyl-CoA mutase, methionine synthase or a protein with at least 60% identity to any one of these proteins or a fragment thereof. The percent identity of two amino acid sequences or of two nucleic acid sequences is determined by aligning the sequences for optimal comparison purposes (e.g., gaps can be introduced in both sequences for best alignment) and comparing the amino acid residues or nucleotides at corresponding positions. The “best alignment” is an alignment of two sequences which results in the highest percent identity. The percent identity is determined by the number of identical amino acid residues or nucleotides in the sequences being compared (i.e., % identity = # of identical positions/total # of positions x 100).

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm known to those of skill in the art. An example of a mathematical algorithm for comparing two sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264-2268, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5877. The NBlast and XBLAST programs of Altschul, et al. (1990) J. Mol. Biol. 215:403-410 have incorporated such an algorithm. BLAST nucleotide searches can be performed with the NBlast program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilised as described in
Altschul et al. (1997) Nucleic Acids Res. 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (Id.). When utilising BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

Another example of a mathematical algorithm utilised for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). The ALIGN program (version 2.0) which is part of the CGC sequence alignment software package has incorporated such an algorithm. Other algorithms for sequence analysis known in the art include ADVANCE and ADAM as described in Torellis and Robotti (1994) Comput. Appl. Biosci., 10:3-5; and FASTA described in Pearson and Lipman (1988) Proc. Natl. Acad. Sci. 85:2444-8. Within FASTA, ktup is a control option that sets the sensitivity and speed of the search.

However, the important factor is that a protein with a similar sequence identity to natural CBP is also functionally the same as the CBP binding proteins. In other words, the proteins produced according to the present invention are able to make a complex with Cbl and bind to the corresponding CBP receptor.

The plant is preferably a “Generally Recognised As Safe” (GRAS) plant. Plants that only produce pollen in the second year of the life cycle such as carrots can be used to prevent cross contamination of other plants in the environment with genetically modified material.

A whole plant can be regenerated from the single transformed plant cell by procedures well known in the art. The invention also provides for propagating material or a seed comprising a cell. The invention also relates to any plant or part thereof including propagating material or a seed derived from any aspect of the invention.
In a second aspect the present invention provides a cobalamin binding protein that is expressed in a transgenic plant, or by transgenic plant cell culture. This protein is preferably isolated or purified from the plant material. This protein can be either in the apo- (i.e. not bound to cobalamin) or holo-form (i.e. bound to cobalamin.) The protein that is produced by the transgenic plant has different carbohydrate moieties attached to the molecule compared to a similar protein produced by humans or micro-organisms such as yeast. The variation in the carbohydrate content of the proteins can be seen by the different apparent molecular weights of the protein produced by the transgenic plant as compared to the protein produced by humans or yeast. Once the proteins are stripped of the carbohydrate groups, all the proteins have the same molecular weight as seen by SDS-Polyacrylamide gel electrophoresis.

In a third aspect the present invention provides a method of isolating and/or purifying the cobalamin binding protein or a functional fragment thereof as defined above comprising the following steps:

(a) Homogenisation of the transgenic plant material;

(b) Filtration of the supernatant formed by centrifugation of the homogenate;

(c) Affinity column chromatography using cobalamin

(d) Elution of the cobalamin binding protein attached to cobalamin

(e) Gel filtration;

And optionally further comprising the following steps to produce the apo-form of the cobalamin binding protein:
(f) Dialysis against 5M guanidine HCL;

(g) Dialysis against 0.2M sodium phosphate.

In a fourth aspect the present invention provides a composition comprising a cobalamin binding protein of the present invention. The cobalamin binding protein can be provided in a substantially pure form. Alternatively, it can be provided as transgenic plant material which is raw, unprocessed transgenic plant material or more or less processed transgenic plant material that is ingested e.g. in the form of chopped, ground, homogenized transgenic plant material, liquid transgenic plant extract as well as partially or completely purified recombinant CBP.

The composition is preferably a pharmaceutical composition. The compositions of the invention may be adapted for administration by any appropriate route, for example by the oral (including buccal or sublingual) route. Such formulations may be prepared by any method known in the art of pharmacy, for example by bringing into association the active ingredient with the carrier(s) or excipient(s).

Pharmaceutical formulations adapted for oral administration may be presented as discrete units such as capsules or tablets; powders or granules; solutions or suspensions in aqueous or non-aqueous liquids; edible foams or whips; or oil-in-water liquid emulsions or water-in-oil liquid emulsions.

Preferred unit dosage formulations are those containing a daily dose or sub-dose, as herein above recited, or an appropriate fraction thereof, of an active ingredient.

It should be understood that in addition to the ingredients particularly mentioned above, the formulations may also include other agents conventional in the art having regard to
the type of formulation in question, for example those suitable for oral administration may include flavouring agents. The recombinant plant CBP may also be delivered in combination with a second component like cobalamin or cobalamin analogs or cobalamin containing a tracer atom (or molecule). Tablets with recombinant plant CBP with or without a second component may also be designed for the release of CBP in the intestine to avoid the low gastric pH and the gastric proteases.

Preferably, the compositions of the invention are presented for oral use. The recommended daily dose for vitamin B12 is 2.5 micrograms which corresponds to 125 micrograms of IF-Cbl or 122.5 micrograms of IF.

In a fifth aspect the present invention provides a foodstuff comprising transgenic plants of the invention or plant material derived from such plants.

In a sixth aspect the invention provides a method for treating cobalamin deficiency comprising administration of a composition that includes at least one cobalamin binding protein of the present invention. The deficiency can be overcome by providing additional amounts of the proteins involved in vitamin B12 uptake and transport. These proteins are preferably any one or more of transcobalamin, haptocorrin and intrinsic factor. The proteins can be administered alone i.e. in the apo-form, or in conjunction with cobalamin or a cobalamin analog i.e. in the holo-form. A “holo-form” of a cobalamin binding protein is a complex of the cobalamin binding protein and its ligand e.g. cobalamin or a cobalamin analog. An “apo-form” of a cobalamin binding protein is the cobalamin binding protein which is ready to bind a cobalamin or a cobalamin analog.
In a seventh aspect the invention provides CBP of the invention for use in diagnostic tests. In such tests the content of cobalamins in for example blood is detected by the ability of the extracted biological sample to compete with tracer Cbl for binding to CBP as described by Nexø and Gimsing, 1981, Scand. J. Clin. Lab. Invest. 41:465-468; “Insolubilized pure human intrinsic factor used for quantitation of cobalamins in serum”. In other tests the purpose is to measure the CBP, either its total content or its holo- or apo-form. Such tests can be performed by for example enzyme linked immunosorbent assay (ELISA) directly (Nexø et al. 2000, Clinical Chemistry 46 (10):1643-1649), or in combination with a pretreatment of the sample that removes the apo-form i.e as described by Nexø, 1975, Biochim. Biophys. Acta, 379:189-192.

In another aspect the present invention provides a method for quantifying cobalamin absorption from the intestine by means of a Schilling test utilizing IF or a functional fragment thereof that has been isolated from a transgenic plant expressing the cobalamin binding protein or a functional fragment thereof. For example, a “Schilling test” may be performed by using a dose of Cbl with radioactive cobalt for oral ingestion followed by 24 hours of urine collection and measurement of the fraction of radioactive Cbl in the urine. If no radioactive Cbl is detected in the urine of this first stage of the test, a second test is performed a week later. Now the test uses a dose of Cbl with radioactive cobalt together with IF for oral ingestion followed by 24 hours of urine collection and measurement of the fraction of radioactive Cbl in the urine (Fairbanks, V.F. Test for pernicious anaemia: the “Schilling test” Mayo Clin Proc. 1983, 58: 541-544).

In a further aspect the invention provides a method for quantifying cobalamin or analogs thereof utilizing the protein of the present invention. The isolated CBP produced by the transgenic plant can be used for example in a competitive binding assay utilizing labeled cobalamin or analogues thereof.
In yet a further aspect the present invention provides a method for quantifying CBP utilizing the protein of the present invention. The CBP of the present invention can be isolated and then labeled radioactively, and used in a competitive binding assay with cobalamin or an analog thereof.

In yet another aspect the present invention provides a method for quantifying receptors for cobalamin binding proteins utilizing the protein of the present invention. For example the rhIF may be complexed with a labeled cobalamin molecule e.g. radioactive cobalamin, to determine the number or binding capacity of the intestinal IF-B12 receptor.

In a further aspect the present invention provides a nucleic acid construct comprising nucleic acid coding for one or more cobalamin binding proteins, operably linked to one or more regulatory sequences capable of directing expression in a plant. The regulatory sequence can, for example, be a promoter such as 35S CaMV. In addition a signal peptide sequence such as the Arabidopsis thaliana extensin signal peptide, Phaseolus vulgaris chitinase signal peptide, or Nicotiana tabacum glucan beta – 1,3-glucanase can also be included. In one preferred embodiment the nucleic acid is DNA.

In yet another aspect the present invention provides a vector comprising a nucleic acid construct of the invention. The vector may be a plasmid, cosmid or phage. Vectors frequently include one or more expressed markers which enable selection of cells transfected, or transformed, with them and preferably, to enable a selection of cells, containing vectors incorporating heterologous DNA. If the vector is intended for expression, sufficient regulatory sequences to drive expression will be present. The nucleic acid and promoter sequences according to the invention are preferably for expression in plant cells.
In yet a further aspect the invention also provides a plant cell comprising a nucleic acid sequence of the invention and/or a vector of the invention. The cell may be termed as a “host” which is useful for manipulation of the nucleic acid, including cloning. Alternatively, the cell may be a cell in which expression of the nucleic acid is obtained, most preferably a plant cell. The nucleic acid can be incorporated into cells by standard techniques known in the art. Preferably, nucleic acid is transformed into plant cells using a disarmed Ti plasmid vector carried in agrobacterium by procedures known in the art, for example, as described in EP-A-0116718 and EP-A-0270822. Foreign nucleic acid can alternatively be introduced directly into plant cells using an electrical discharge apparatus or by any other method that provides for the stable incorporation of the nucleic acid into the cell. Nucleic acid of the present invention preferably contains a second “marker” gene that enables identification of the nucleic acid. This is most commonly used to distinguish the transformed plant cells containing the foreign nucleic acid from other plant cells that do not contain the foreign nucleic acid. Examples of such marker genes include antibiotic resistance, herbicide resistance and glucuronidase (GUS) expression. Expression of the marker gene can be controlled by a second promoter, which allows expression of the marker gene in all cells.

The foreign nucleic acid is preferably introduced into only the mitochondria and/or the chloroplasts of the host cell. The nucleic acid may be become introduced into the genome, within the nucleus, or in other organelles, including mitochondria or plastids such as protoplastids, chromoplasts or leucoplasts. Pollen does not contain plastids or mitochondria. Therefore, by only having the foreign nucleic acids within these organelles, this will prevent the transfer of transgenic material to wild type plants in the surrounding environment by cross pollination.
The aim of the present invention is to produce recombinant cobalamin binding proteins (CBP) in plants. Plants do not contain or use cobalamin and therefore the recombinant CBP expressed in plants will all be in the apo-form (not complexed with cobalamin) whereas CBP from natural sources are a mixture of holo- and apo-form CBP. Unlike use of transgenic plants, production of a particular CBP from natural sources involves further purification from other CBPs. Furthermore, transgenic plants provide a cheap method for large scale production of CBP, as compared to all other known sources of the proteins.

The present invention will be easy to scale-up for production of large quantities of recombinant CBP by simply planting larger areas of land with the CBP producing plants. This is relatively cheap compared to production of CBP in high technology demanding fermentors and incubators when yeast or insect cells are used.

The use of natural sources to isolate CBP has some limitations. Because of the relatively low concentrations of TC in blood and milk this protein is very expensive for isolation in large scale. Isolation of IF is performed from porcine stomach in large scale and to a lesser extent from rat stomach and human stomach juice. Since HC is also present in the stomach, a high degree of purification is needed to separate these CBP when IF is used for diagnostic kits. For example, IF free of Cbl is requested (FDA requirement) for use as a binding protein in assays of Cbl in plasma. In addition to these problems natural sources also constitute a serious risk for transmission of diseases to patients receiving the CBP. Expression of recombinant CBP in transgenic plants will result in extracts with no other CBP than the recombinant CBP. Furthermore the risk of transmitting animal or human diseases from natural sources of CBP to patients is eliminated by the use of CBP from transgenic plants. Plant expression systems also give the opportunity to use GRAS (Generally Recognized As Safe) organisms as host for the production of CBP.
As examples of the use of transgenic plants as an expression system for production of CBPs, we have expressed human IF and human TC in the plant *Arabidopsis thaliana*. We tested the transgenic plants for cobalamin binding capacity (CBC) as described by (14).

The description of the invention hereafter refers to *Arabidopsis thaliana*, when necessary for the sake of example. However, it should be noted that the invention is not limited to genetic transformation of plants such as *Arabidopsis thaliana*. The method of the present invention is capable of being practiced for other plant species, using methods/techniques well known to those skilled in the art.

The invention will now be described with reference to the following examples, which should not be construed as in any way limiting the invention.

The examples refer to the figures in which:

**Figure 1** shows the nucleotide sequence encoding extensin signal peptide sequence fused to the mature human intrinsic factor encoding region and part of the 3'-untranslated region. This nucleotide sequence is a fusion of a nucleotide sequence (position 7-119) adopted from GenBank accession no. AF104327 which encodes an *Arabidopsis thaliana* extensin-like signal peptide with the amino acid sequence MASSSIALFLALNLFFTTISA. The methionine start codon (ATG) of this signal peptide sequence is underlined. The nucleotide sequence encoding mature human intrinsic factor (position 120-1316) is shown in bold letters and adopted from GenBank accession no X76562. This sequence is followed by a translational stop codon (TAA) which is underlined and a nucleotide sequence from the 3'-untranslated region of the
intrinsic factor mRNA. The underlined restriction sites XbaI (position 1-6) and XmaI (position 1425-1430) was introduced to facilitate cloning in the plant transformation vector.

**Figure 2** shows the amino acid sequence of mature human intrinsic factor which was encoded by the nucleotide sequence shown in bold letters in figure 1 at position 120-1316.

**Figure 3** shows a nucleotide sequence at position 7-129 from Phaseolus vulgaris CH5B-chitinase, GenBank accession no. S43926 and it encodes a signal peptide with the amino acid sequence MKKNRMIMICSVGVVWMLLVGGSYG. This nucleotide sequence was fused to the nucleotide sequence shown in bold letters in figure 1 that encodes the mature human intrinsic factor. The XbaI restriction site used for cloning is underlined at position 1-6 and the translational start codon ATG is underlined at position 52-54.

**Figure 4** shows a nucleotide sequence at position 7-144 from *Nicotiana tabacum* glucan beta-1,3-glucanase gene, GenBank accession no. M60402 and it encodes a signal peptide with the amino acid sequence MSTSHKHNTPQMAITLTLLGVVASSIDIGA. This nucleotide sequence was fused to the nucleotide sequence shown in bold letters in figure 1 that encodes the mature human intrinsic factor. The XbaI restriction site is underlined (position 1-6) and the translational start codon ATG is underlined at position 49-51.

**Figure 5** shows the nucleotide sequence encoding the extensin signal peptide sequence fused to the mature human transcobalamin encoding region. This nucleotide sequence is a fusion of a nucleotide sequence (position 7-119) adopted from GenBank accession no. AF104327 which encodes an *Arabidopsis thaliana* extensin-like signal peptide with the
amino acid sequence MASSSIALFLALNLFPFTTISA. The methionine start codon (ATG) of this signal peptide sequence is underlined. The nucleotide sequence encoding mature human transcobalamin (position 120-1346) is shown in bold letters and adopted from GenBank accession no NM_000355. This sequence is followed by a translational stop codon (TAG) at position 1347-1349 which is underlined. The underlined restriction sites XbaI (position 1-6) and Xmal (position 1350-1355) was introduced to facilitate cloning in the plant transformation vector.

Figure 6 shows the amino acid sequence of mature human transcobalamin which was encoded by the nucleotide sequence shown in bold letters in figure 5 at position 120-1346.

Figure 7 shows the native intrinsic factor (o) present in human gastric juice and recombinant human intrinsic factor extracted from transgenic plants of Arabidopsis thaliana (●) which were compared concerning their ability to bind cobalamin or cobinamid. Equal amounts of the respective proteins were added to a mixture containing a fixed amount of cobalt (57Co) labelled cobalamin mixed with increasing amounts of non-radioactive cobalamin or cobinamide (X axis). Free and bound ligand were separated and the amount of 57Co attached to the protein was measured in a gamma counter. The 57Co fraction bound relative to the amount of 57Co bound in the absence of unlabeled cobalamin or cobinamide was calculated (Y axis). The figure shows that recombinant IF behaves as does native IF with specificity for binding cobalamin but not cobinamid.

Figure 8 shows how polyclonal antibodies against human gastric intrinsic factor and alkaline phosphatase-conjugated immunoglobulins were used to visualize intrinsic factor on a Western blot containing proteins separated by SDS-PAGE. Transgenic
Arabidopsis thaliana plants expressing recombinant human intrinsic factor were harvested and homogenized in 0.2 M phosphate buffer before centrifugation and analysis of the supernatant. For comparison transgenic yeast (Pichia pastoris) containing an insert of human intrinsic factor was used for expression of recombinant human intrinsic factor. Secreted proteins form the fermentation media were precipitated with ammonium sulfate (80% w/v) before dialysis against 20 mM Tris pH 8.0 and analysis. Purified human gastric intrinsic factor was also used for comparison. An aliquot of each sample was treated with the enzyme PNGaseF to remove carbohydrate from the proteins. Lane: “Plant IF” contains the proteins from the plant extract; “Plant IF + PNGaseF” contains the proteins from the plant extract treated with PNGaseF; “Yeast IF” contains yeast protein; “Yeast IF + PNGaseF contains yeast protein treated with PNGaseF; “Human gastric IF” contains purified human IF and “Human gastric IF + PNGaseF” contains purified human IF treated with PNGaseF. The arrow marks the 45 kDa band with deglycosylated IF from the three samples treated with PNGaseF.

The blot shows that the glycosylated form of IF from the three samples have significant differences in molecular weight but the deglycosylated samples contain mature IF with the same molecular weight.

**Figure 9** compares the glycosylation and molecular weight of intrinsic factor prepared from various sources. PAS staining was used to visualize glycoproteins. Recombinant human intrinsic factor (rhIF) from transgenic plants (Arabidopsis thaliana) and transgenic yeast (Pichia pastoris) were isolated and purified by affinity chromatography on a column with cobalamin. Purified human gastric IF was used for comparison. Bovine PAS-3 is heavily glycosylated and function as a control for PAS staining. Bovine serum albumin (BSA) has no glycosylation and function as a “negative” control for PAS staining. The samples were run in SDS-PAGE in duplicate.
gels. The second gel was stained with coomassie brilliant blue. The gels show that recombinant plant IF is glycosylated since an approximately 50 kDa rhIF band from transgenic plants is stained with PAS.

**Figure 10** shows the purification method for isolating rhIF and a blot of the protein fragments used for N-terminal sequencing. Purified recombinant human intrinsic factor (rhIF) was run by SDS-PAGE, blotted onto a PVDF-membrane and stained with coomassie brilliant blue. The mature rhIF and the partially cleaved rhIF fragments (proteases present in Arabidopsis thaliana cleave rhIF) were analyzed by amino acid sequencing. N-terminal sequencing of the three fragments marked with arrows showed that the approximately 50 kDa fragment has the same N-terminus as mature human gastric intrinsic factor. The lower two fragments (30 and 20 kDa) are a result of a proteolytic split in front of amino acid residue 285 of mature hIF.

**Figure 11** shows the expression of transcobalamin in transgenic plants. Polyclonal antibodies against human transcobalamin (TC) and alkaline phosphatase-conjugated immunoglobulins were used to visualize TC on a Western blot containing proteins separated by SDS-PAGE. Transgenic Arabidopsis thaliana plants (no 9-11) expressing recombinant human TC were harvested and homogenized in 0.2 M phosphate buffer before centrifugation and analysis of the supernatant. The blot shows a single protein with the expected molecular size of 45 kDa in each of the three plants

**Figure 12** shows the ability of plant recombinant human transcobalamin (o) and plant recombinant human intrinsic factor (•) to bind cobalamin or cobinamid. Both recombinant proteins were extracted from transgenic Arabidopsis thaliana plants. Equal amounts of the respective proteins were added to a mixture containing a fixed
amount of cobalt (57Co) labeled cobalamin mixed with increasing amounts of non-radioactive cobalamin or cobinamide (X axis). Free and bound ligand was separated and the amount of 57Co attached to the protein was measured in a gamma counter. The 57Co fraction bound relative to the amount of 57Co bound in the absence of unlabelled cobalamin or cobinamide was calculated (Y axis). The figure shows that recombinant human intrinsic factor and recombinant human transcobalamin both bind cobalamin but not cobinamid.

**Figure 13** shows the binding of intrinsic factor to the human intestinal receptor protein cubilin. The binding between intrinsic factor and its receptor cubilin was analysed employing a BIAcore 2000 instrument (Biacore AB, Uppsala, Sweden). An increase in Resp. Diff. (Y axis) indicates binding between intrinsic factor and cubilin. At time approximately 100 a solution with intrinsic factor is added to the immobilized cubilin. Intrinsic factor was isolated from transgenic plants, human gastric juice and hog stomach. Preparations of vitamin B12 saturated and vitamin B12 unsaturated intrinsic factor were used. At time approximately 600 intrinsic factor is removed from the solution and the dissociation between cubilin and intrinsic factor is followed. The results indicate that recombinant intrinsic factor – like native intrinsic factor – binds to cubilin only when saturated with vitamin B12 and that the binding characteristics for recombinant and native intrinsic factor are alike.

**Example 1**

As shown in Figure 1, the extensin signal peptide encoding nucleotide sequence from *Arabidopsis thaliana* was fused to the nucleotides encoding mature human intrinsic factor (Figure 2). This construct was inserted in the plant transformation vector CRC-179.
Construction of the CRC-179 vector

The vector pPZP 211 (Hajdukiewicz, P; Svab, Z; & Maliga, P. 1994 Plant Mol. Biol. 25, 989-994) was digested with EcoRI and KpnI and a pAnos sequence was released from pGPTV KAN (Becker, D; Kemper, E; Schell, J & Masterson, R. 1992 Plant Mol Biol 20, 1195-1197) by the same set of enzymes and cloned into the pPZP 211 vector. The resulting vector was digested with PstI and KpnI and blunt-ended. A blunt-ended EcoRI and HindIII fragment containing the 35S CaMV promoter from the vector described in Jefferson, RA; Kavanagh, TA & Bevan, MW. 1987 EMBO J. 6, 3901-3907 was cloned into the blunt-ended vector. This vector was named CRC-179.

The bacteria *Agrobacterium tumefaciens* was transformed with this recombinant vector.

Culture of *Agrobacterium tumefaciens*

The *Agrobacterium tumefaciens* strain used was GV3101 (pMP90) (Koncz and Schell, 1986) carrying the binary plasmid CRC-179 with an insert encoding a CBP cloned into the XbaI-Xmal sites. The insert sequences are shown in the figures: 1, 3, 4, & 5. The bacteria were grown to stationary phase in 200 ml liquid culture at 28-30°C, 250 rpm in sterilized LB media (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter water) carrying added rifampicin (100 mg/ml), streptomycin (100 mg/ml) and gentamycin (50 mg/ml). Cultures were started from a 1:200 dilution of a smaller overnight culture and grown for 16-18 hours. Bacteria cells were harvested by centrifugation for 10 min at 5500 g at room temperature and then resuspended in 400 ml inoculation medium (10 mM MgCl₂, 5 % w/v sucrose and 0.05 % v/v Silwet L-77 (Lehle Seeds, Round Rock, TX, USA)).

The recombinant *A. tumefaciens* bacteria were used to transform *Arabidopsis thaliana* plants.
Transformation of *Arabidopsis* plants

The *Arabidopsis* plants were transformed by the floral dip method (Clough and Bent, 1998).

**Plant growth**

*Arabidopsis* plants (ecotype Col-0) were grown to flowering stage in growth chamber, 20 °C day/18°C night with LiCl lighting for 18 h/day, humidity 70%. Between 20 and 25 plants were planted per 64 cm² pot in moistened soil mixture consisting of: 40 kg soil orange and 40 kg soil green (Stenrøgel Mosebrug A/S Kjellerup, DK), 25 liter 4-8 mm Fibroklinker (Optiroc, Randers, DK), 12 liter Vermiculite (Skamol, DK) and 300 g Osmocote plus NPK 15-5-11, 3-4 months (Scott's, UK).

To obtain more floral buds per plant, inflorescences were clipped after most plants had formed primary bolts, relieving apical dominance and encouraging synchronized emergence of multiple secondary bolts. Plants were dipped when most secondary inflorescences were about 7-13 cm tall (7-9 days after clipping).

The transgenic *Agrobacterium* suspension was added to a 400 ml beaker and plants were inverted into the suspension such that all above-ground tissues minus the rosette were submerged. The plants were removed after 10-15 sec of gentle agitation and placed in horizontal position in a sealed plastic bag for 24 hours at room temperature.

After 24 hours the plants were moved to the growth camber and the plastic bags were removed. Plants were grown 3-4 weeks until siliques were brown and dry. Seeds were harvested and allowed to dry at room temperature for 7 days.

**Selection of transformants**
Seed were surface sterilized by a treatment with 0.5 % sodium hypochlorite containing 0.05 % v/v Tween 20 for 7 min, then with 70 % ethanol for 2 min, followed by three rinses with sterile water.

To select for transformed plants the sterilized seeds were plated on kanamycin selection plates at a density of approximately 2000 seeds per 144 cm$^2$ and grown for 8-10 days at 21 °C under light for 16 hours per day. Selection plates contained 1 x MS medium (Duchefa, Haarlem, NL #M 0222), 1 % w/v sucrose, 0.9 % w/v agar noble (Difco, Detroit, USA), 50 mg/ml kanamycin, pH 5.7. After selection the transformed plants were transferred to growth chambers (see Plant growth).

Seeds from these infected plants were planted and recombinant plants were identified by western-blotting analysis. Seeds from recombinant plants were used to grow new recombinant plants called IF-plants.

One kilogram of three week old IF-plants was homogenization with 2 liters of phosphate buffer and clarified by centrifugation. This extract contained 100 mg recombinant human IF with CBC. This IF had specificity for cobalamin binding whereas the analog cobinamid was not bound by the IF as tested by the method described by (15). Figure 7 shows that the rhIF from plants and native human gastric IF have the same specificity for binding Cbl, but not cobinamid.

The transgenic plants were analyzed as described by (16) and shown to contain no cobalamin. This shows that the plant rhIF was at the apo-form. Analysis of the protein from these transgenic plants showed that they express a protein of approximately 50 kDa as recognized on a Western-blot with antibodies against human IF (see fig 8,10). Amino acid sequencing of the N-terminal region of this 50 kDa protein showed the same
sequence as that present in mature natural IF (figure 2). Therefore, the post-translational cleavage of the extensin signal peptide from the fusion protein results in the secretion of a recombinant IF with the correct N-terminus. The size of approximately 50 kDa indicate that the protein was full-length. A mature full-length IF is 399 amino acids with a calculated molecular weight of 43412 Da. The recombinant IF contains some carbohydrates as shown by PAS-staining of blotted recombinant protein separated by SDS-PAGE (see fig 9). Deglycosylation of rhIF from plants results in a protein with an approximately similar observed and calculated molecular weight (Figure 8). The molecular size of natural human IF was approximately 5 kDa larger than the recombinant plant IF as estimated from the western-blot. Some difference in the carbohydrate composition is expected between natural human IF and recombinant plant IF since carbohydrate composition is tissue specific and to some extent unique to each individual. Differences in the molecular weight between natural and recombinant IF may therefore be a result of different carbohydrate composition. This was shown by removal of the carbohydrates from human gastric IF, human IF expressed in yeast and human IF expressed in plants. The deglycosylated form of these three IF proteins to have the same molecular weight (see fig 8).

On a Western-blot containing purified rhIF from transgenic Arabidopsis thaliana, two minor bands of approximately 30 and 20 kDa were observed in addition to the 50kDa band with mature rhIF (figure 10). N-terminal sequencing of these two bands showed, that the 30 kDa band contained the N-terminal sequence of mature human IF. The 20 kDa band contained an N-terminus located at glycine-285 of mature IF. The calculated molecular weight of the fragment containing amino acid residues 1-284 was 30612 Da and the fragment containing amino acid residues 285-399 had a calculated molecular weight of 12817 Da. The inconsistency between the observed 20 kDa and the calculated 12817 Da most likely was a result of glycosylation of one or more of the four potential
N-linked glycosylation sites of the fragment containing amino acid residue 285-399. The PAS-staining also recognized the 20 kDa band showing that some glycosylation was present on the fragment. No PAS-staining was observed for the 30 kDa fragment although one potential N-linked glycosylation site was present on this fragment. This is consistent with that no difference was found between the observed and calculated molecular weight for the fragment containing amino acid residues 1-284.

Figure 13 shows that rHIF from plants in complex with vitamin B12 binds to the human intestinal receptor protein cubulin. The apo-form of rHIF does not bind to the cubulin receptor. For comparison binding of human gastric IF and hog IF were also shown to bind to the cubulin receptor only when in holo-form. These results show, that recombinant human intrinsic factor from plants behaves as native gastric intrinsic factor with respect to receptor binding.

Example 2

*Arabidopsis thaliana* was transformed with a vector construct which contained a nucleotide sequence encoding the signal peptide from the *Phaseolus vulgaris* chitinase CH5B fused to the nucleotide sequence encoding mature human intrinsic factor (figure 3). This construct was used to generate transgenic *Arabidopsis thaliana* plants. These plants were shown to contain CBC at the same level as most of the extensin-IF plants showing that the choice of signal peptide for intrinsic factor expression is not restricted to one sequence.

Example 3

*Arabidopsis thaliana* was transformed with a vector construct which contained a nucleotide sequence encoding the signal peptide from the *Nicotiana tabacum* glucan beta-1,3-glucanase fused to the nucleotide sequence for mature human intrinsic factor.
(Figure 4). This construct was used to generate transgenic *Arabidopsis thaliana* plants. These plants were shown to contain CBC at the same level as most of the extensin-IF plants showing that the choice of signal peptide for intrinsic factor expression is not restricted to one sequence.

**Conclusions from IF-Plants in examples 1-3**

As far as we have tested the recombinant human IF from plants it behaves as natural human gastric IF concerning its mature N-terminus, recognition by anti-IF antibodies, binding of cobalamin, lack of cobinamide binding, binding to the intestinal receptor, and presence of carbohydrates. In contrast to gastric juice where IF is present together with another CBP, haptocorrin, and to some extent cobalamin from the food, transgenic IF-plants contain no cobalamin or other CBP than IF. The glycosylation of IF from transgenic plants was different from human gastric IF. Another difference between IF from human gastric mucosa and IF from transgenic plants is that IF from plants is in the apo-form whereas IF from human beings is a mixture of the apo- and holo-form.

**Example 4 - TC-plants**

Extracts from one kilogram of transgenic *Arabidopsis thaliana* (TC-plants) transformed with an extensin-transcobalamin construct (Figure 5) contained 20 mg of recombinant human TC with CBC. Western blot analysis showed a single band of approximately 45 kDa which reacted with antibodies against human TC (Figure 11). The calculated molecular weight of mature TC (figure 6) with 409 amino acid residues is 45536 Da, showing that the observed and calculated molecular weights are similar. These results show that a plant expression system is able to produce recombinant human TC of the expected size and with CBC.
The transgenic plants were analyzed as described by (16) and shown to contain no cobalamin. This shows that the recombinant human TC was at the apo-form. As for rhIF, rhTC obtained from plants binds to Cbl but not cobinamid (Figure 12). N-terminal amino acid sequencing showed that the extensin signal peptide was removed from the secreted rhTC genertaing the normal mature N-terminal found in native human transcobalamin.

**Example 5. Purification of the recombinant intrinsic factor from plants**

1 kg of the crude plant material was chopped and homogenized in 2 L of 0.2 M Sodium Phosphate buffer, pH 7.5. The homogenate was centrifuged at 4000 rpm for 10 min and filtered through Watman paper on a Buchner funnel. The filtrate can be stored frozen at that stage. Intrinsic factor was adsorbed from the solution on an affinity matrix according to a previously described method (Nexø, E., 1975 Biochim. Biophys. Acta 379, 189-192). After elution from the column intrinsic factor (saturated with cobalamin = holo-form) was subjected to gel filtration, dialyzed against water and lyophilized. Preparation of cobalamin unsaturated intrinsic factor (= apo-form) required an additional step: dialysis against 5 M guanidine HCl for two days followed by dialysis against 0.2 M Sodium Phosphate buffer, pH 7.5.
REFERENCES


19 Nexø et al. 2000, Clinical Chemistry 46 (10):1643-1649

CLAIMS

1. A transgenic plant that expresses at least one protein capable of binding cobalamin and/or analogs thereof.

2. A transgenic plant as claimed in claim 1 wherein the protein is any one of transcobalamin, intrinsic factor, haptocorrin, methylmalonyl-CoA mutase, methionine synthase or a protein with at least 60% sequence identity to any one of these proteins or a functional fragment thereof.

3. A transgenic plant as claimed in claim 1 or claim 2 wherein the plant is “Generally Recognised As Safe” (GRAS.)

4. Propagating material derived from a transgenic plant as defined in any one of claims 1 to 3.

5. Propagating material as claimed in claim 4 which comprises seeds.

6. A cobalamin binding protein or a functional fragment thereof that is expressed in a transgenic plant as defined in any one of claims 1 to 3.

7. A cobalamin binding protein or a functional fragment thereof as claimed in claim 6 which has been isolated and/or purified from the transgenic plant.

8. A method of isolating and/or purifying the cobalamin binding protein or a functional fragment thereof as defined in claim 6 which includes the step of:

   Homogenising the transgenic plant material.
9. A method as claimed in claim 8 which comprise the following steps:

Filtration of the supernatant formed by centrifugation of the homogenate;

Affinity column chromatography using cobalamin

Elution of the cobalamin binding protein attached to cobalamin

Gel filtration;

Dialysis against water;

And optionally further comprising the following steps to produce the apo-form of the cobalamin binding protein:

Dialysis against 5M guanidine HCL;

Dialysis against 0.2M sodium phosphate.

10. A composition comprising a cobalamin binding protein or a functional fragment thereof as defined in claim 6 or claim 7.

11. A composition as claimed in claim 10 which is a pharmaceutical composition and optionally further comprises one or more pharmaceutically acceptable diluents, excipients and/or carriers.

12. A composition as claimed in claim 10 or claim 11 which is for oral use.

13. A foodstuff comprising transgenic plants as defined in claims 1 to 3 or plant material derived from said plants.
14. The use of a protein as defined in claim 6 or claim 7 in diagnostic tests.

15. A method for quantifying cobalamin absorption from the intestine by means of a Schilling test utilizing a protein as defined in claim 6 or claim 7, wherein the protein is Intrinsic factor or a functional fragment thereof.

16. A method for quantifying cobalamin and analogs thereof utilizing a protein as defined in claim 6 or claim 7.

17. A method for quantifying a cobalamin binding protein utilizing a protein as defined in claim 6 or claim 7, or antibodies against said protein.

18. A method for quantifying receptors for a cobalamin binding protein utilizing a protein as defined in claim 6 or claim 7.

19. A method as claimed in claim 18, further utilizing labeled cobalamin.

20. A method as claimed in claim 19, wherein the cobalamin is radioactively labeled.

21. A nucleic acid construct comprising nucleic acid coding for one or more cobalamin binding proteins, operably linked to one or more regulatory sequences capable of directing expression in a plant.

22. A nucleic acid construct as claimed in claim 21 wherein the nucleic acid is DNA.

23. A nucleic acid as claimed in claim 21 or claim 22 wherein the regulatory sequences are from any one of Arabidopsis thaliana extensin signal peptide, Phalseolus vulgaris chitinase signal peptide, Nicotiana tabacum glucan beta – 1,3-glucanase and/or 35S CaMV promoter.
24. A vector comprising a nucleic acid construct as defined in any one of claims 21 to 23.

25. A plant cell comprising a vector as claimed in claim 18 or claim 24 or nucleic acid construct as defined in any one of claims 21 to 23.

26. A whole plant, or part thereof comprising a plant cell as defined in claim 25.

27. A plant cell as claimed in claim 25, or a plant, or part thereof as claimed in claim 26, wherein the vector or nucleic acid construct is within the nucleus, plastids and/or mitochondria.

28. A method for treating cobalamin deficiency comprising administering a protein or a functional fragment thereof as defined in claim 6 or claim 7 or a pharmaceutical composition as defined in claim 11 or claim 12 to a subject in need thereof.

29. A method as claimed in claim 28 wherein the cobalamin deficiency is caused by low levels of at least one of intrinsic factor, transcobalamin and haptocorrin.
TCTAGAACTCACAACCTAGCTAGCTAGTAAACAGTAGTTTTTCTATATACCA 50
AAAATTGGCTTCAAGTTCCATAGCCTTTTTCTTTGGCTCTCAATCTTCTT 100
TTTCAACAATCTCCGGCCAGTAGCCACCCAGCCAGTTCTAGCTCCGTTT 150
CTCCAGCAGAGGACCCCTTGGAATGGAATACGAATCTAGTCCAGGAAAC 200
TCGGTGAACCTTCACTACGCCCTCACCACCCACGACATCTTGGATGCGATGA 250
TCTGGCCGAGGCTCAACACTTGAGGCACACCTCAGCTCTGGACTCTACCCAGC 300
TCATGTCCACGGCAACAACGATCTAAAACTTTGCGGAGCTGCGGCTCACC 350
ATCATGGGCCCTCAGCTCCCTCCTGCGCAGACCCCTGGGATAAGATGATCAT 400
TCTACAAAGACAAATGGGAGAGCTGGGCACCTCCAGCCCAACACGCTGAAG 450
CATCAGCCTCTATAGGGCCAGCTCTAGCGATCTTGGACTGTGGCGAGGAAG 500
AATCTGTGAGCGACCTTGGCCGATAGCGTCCGCTTTTGGGAAAGACACCTGCT 550
GGCCAACACTCCTCTCCCTCAATGTAGACACAGAGCAATGGCACACCTTG 600
CTCTGACCTGTATGTAACACAAGATCCCTGTTAGGTACAGGAGAAATGTTAC 650
AGATCCCTGTGTTGTGTACGACTAAGGATATTGTTGGAAATAATCGACAT 700
GAAGATCAAAGATAATGGGACTCACATTTGGAGACATCTACAGCTAAGCTGCG 750
CCATGGCAGGCTCTCTCTGTCAACACCTTAGGCGCACCCTCTACCAAAGAATGGG 800
TGCAAGAGACTACGGATATGTGATACTCAGAGATAGATGAGAGCGAGGGAATT 850
CCACAACCCCATGTCCAGTACAAATCTCTCCCTCCCTGGGAAGGCAAGA 900
CATACCTAGGTGGCCAGGGTCACGTGTATGTCTTCTGATCTAGGAGGTCAAA 950
CCAACTCTATCCACGAAACCCCTGCGCCTGGCCACCCTCTCTAGCTAACAT 1000
CAGCTGTGTTATACCAACCAAATAACCAGGCTAGGGGGGTTGAGCTGCTCT 1050
TCAACGAGACCATAATGTAGTTAGTGAAAGTGGTGTCAGTCTTTGTT 1100
GTCTAGAAGGAAGCACAGCGCAAATAATCTATGTCTAAATTTGGAACAC 1150
AATGACATCTTGGGGCTGTGCTCTTTCTATCAACAAATTGCAGGAA 1200
AGTTAATCAAGACATACGCTGCTTTTCTATAGTTGGTGAAACACTTT 1250
AATGAAAGGGGTTGCTGACTACATAACCTTCAACCCAGGCAAAACATACAGC 1300
CAATTTCACAGACTAATAAGAGAGGTGGGGTTCACTTTATCAAAACAT 1350
CTCCAAAGGATGGGTGAAATTTTTTCCACCTTCAATTTAAATCTATGCAA 1400
AAAGCGATGCGCTGATGCTAGGTACCCCGG 1430

FIG. 1
SUBSTITUTE SHEET (RULE 26)
FIG. 2

FIG. 3

FIG. 4
TCTAGAACTCAACACCTAGCTAGCTAGTAAACAGTATTTTCTATATACCA
AAAATGGCTTCAAGTTCACATAGCTCTTTTTTGTGCTCTCAATCTCTCTT
TTTCAACAATCTCCGCCAGATGTGTGAATACCCAGAGATGGGACAGCC
ATCTGGTGAAGATGTTGGGCCAGCACCCTCTTTACCTTTGAGTGGACGGCT
TCCCTGGAGCATTTGAACCCACAGACTATGTGGTGGGCTACGCCTCTCCAG
TCTGCAAGGGCTGGGACAAAGGAAGACCTCTACCTGACAGCCTCAAGCTTG
GTTACAGCAGTGGCCTCTAAGGTGTGCTGCGCTCTAGCGGAGGATGACGGTAC
TGCCAGGGCAAGCCTCCATGGCCAGCTGGCCCTCTACCTGCTGCTGCTC
CAGGCCCAACTGTTAGTTTGTCAGGGCCCAAGGGGACAGCTGCTCTCTAGCT
CACAGCTCAAAATGTGGCTCTGGAGATGAGAGAGAGCATTGGCCATGAT
CACAAGGGCCACCCCAACACTAGCTACTACACAGATGTGGCGTGGCATTCT
GGCCCTGTGCTCTCCACAGAAGCGGTCTCAGTTAAGGCTGAGAACAAC
TTCTGTATGCTGTTGGAACCTTTCCACCAGGGCACCATTCTGTGGGACAACA
GCAGCCATGCGGCTTGGCATTCACTGCTGTAAGCGCTCAAAACTTCAA
CCCTGGTCGGGACACAACGATCACCACTGGGCACTGCAAGAACTGCGAGAG
AGCTCTGGAAGCCAGACCCCGCGAGGCGACCTTTGGGAAATGCTACAGC
ACCCCATTTGGCAGTCTAGTTCCTCTGACTGACTCTCCATGGGGCAGA
ACTGGGAAACAGCATGTCTCAAGGGGAGGTTTGGCTTTTGCTGCGCAAGCTG
AGATGGAGCCTCCAGAAATGTCTCTAGATTTCCCCAGCTGCTGCCCGTT
CTGAAACCAACAGACCTACATTGAGCTGAATCTCCAGACCCCAAGAGA
ACGAGTCATGTGGGAACAGCTGTCGAGACAATTCCTCTAGACACCCAAAGAGA
TCATACGTGGTCCAGGCTGCGGTCTAGTATCTCTTTGGGGCGCTACAGACAG
TCATCTCTGCTGCTGGCGGCGTCACACCCAGGAGATGCTCTGAGAAAGGCG
CCATGAGTGAAGGAAGATTCACATATGGAACACAGGCCTCTTTGCTAGGCC
CTTACGTAAACTCCGCAGTGAGGGGAAACCGGCGGCGGAAAGGAGATTCTGG
CAGCTTCTCCGGACACCCCAACTAGGTGCAAAGTATTGCTGACTA
CAGAACCAGGATGGAGAACCATTGAGCTGAGGTGGTATTGCTGGTACGC
CGGGG

FIG. 5
FIG. 6

FIG. 7
FIG. 8
FIG. 9

BSA
PAS-3 (bovine milk)
hIF (gastric)
rhIF (plant)
rhIF (yeast)
Size marker

BSA
PAS-3 (bovine milk)
hIF (gastric)
rhIF (plant)
rhIF (yeast)
Size marker

50 36 30 16 kDa
Raw material

↓

Homogenisation

↓

Centrifugation

↓

B12 column

↓

Elution

[Diagram showing molecular weights and bands labelled: pre-rhIF, Mature rhIF 50 kDa, rhIF 30 kDa, rhIF 20 kDa]

FIG. 10
hTC expressed in Arabidopsis thaliana

FIG. 11

FIG. 12
Binding of Intrinsic factor to its receptor Cubilin

Human recombinant Intrinsic factor (plants) unsaturated (lower curve) and saturated (upper curve) with vitamin B12.

Human Intrinsic factor purified from gastric juice and saturated with vitamin B12. This product is not available unsaturated with vitamin B12.

Hog Intrinsic factor unsaturated (lower curve) and saturated (upper curve) with vitamin B12.

FIG. 13