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Expression of the human milk protein β-casein in transgenic potato plants

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A 1177 bp cDNA fragment encoding the human milk protein â-casein was introduced into *Solanum tuberosum* cells under control of the auxin-inducible, bidirectional mannopine synthase (*mas*19,29) promoters using *Agrobacterium tumefaciens*-mediated leaf disc transformation methods. Antibiotic-resistant plants were regenerated and transformants selected based on luciferase activity carried by the expression vector containing the human â-casein cDNA. The presence of human â-casein cDNA in the plant genome was detected by PCR and DNA hybridization experiments. Human â-casein mRNA was identified in leaf tissues of transgenic plants by RT-PCR analysis. Human â-casein was identified in auxin-induced leaf and tuber tissues of transformed potato plants by immunoprecipitation and immunoblot analysis. Human â-casein produced in transgenic plants migrated in polyacrylamide gels as a single band with an approximate molecular mass of 30 kDa. Immunoblot experiments identified approximately 0.01% of the total soluble protein of transgenic potato leaf tissue as â-casein. The above experiments demonstrate the expression of human milk â-casein as part of an edible food plant. These findings open the way for reconstitution of human milk in edible plants for replacement of bovine milk in baby foods for general improvement of infant nutrition, and for prevention of gastric and intestinal diseases in children.

Keywords: human â-casein; potato; transgenic plants

Introduction

Human milk contains 0.9% protein (9.0 g l^{-1}) of which the casein fraction comprises 20% (1.8 g l^{-1}) (Miller *et al.*, 1990). Human casein consists mainly of two subclasses, â- and κ-caseins. The â-casein is the most abundant (70%) while the κ-casein accounts for only 27% of total casein content (Kunz and Lönnerdal, 1990). Recently, the human counterpart of $Æ_{s1}$-casein has been purified and characterized (Rasmussen *et al.*, 1995). Native â-casein is a 25 kDa protein which exists in several isoforms owing to different levels of phosphorylation (Greenberg *et al.*, 1984; Hansson *et al.*, 1993). *In vivo*, from none to five phosphate groups are attached to serine and threonine residues within the first 10 amino acids of the N-terminal end of the peptide chain (Greenberg *et al.*, 1984). The phosphorylated seryl and threonryl residues facilitate â-casein binding to divalent metal ions such as Ca^{2+}, Zn^{2+}, Mn^{2+} and Fe^{2+} (Dickson and Perkins, 1971; Hansson *et al.*, 1993). The binding of Ca^{2+} to the phosphates in casein aids in the formation of large casein aggregates, which are precipiated by κ-casein (Blakeborough *et al.*, 1983). Phosphorylation of â-casein is essential for both assembly of stable micelles and for the behaviour of casein as a nutrient.

Human â-casein has both nutritional and physiological benefits for human infants. Similar to other milk proteins, such as lactoferrin, IgA and lysozyme, diverse biological effects have been ascribed to â-casein, or â-casein fragments. These effects include enhancement of calcium absorption (Naito and Suzuki, 1974; Mykkanen and Wasserman, 1980), inhibition of angiotensin I-converting enzymes (Kohmura *et al.*, 1989), opiate agonist effects (â-casomorphin peptides) (Brantl, 1984), immunostimulating and modulating effects (Migliori-Samour and Jolles, 1988) and antibacterial functions (isracidin) (Lahov and Regelson, 1996).

Mother's milk is recommended by the nutritional and

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pediatric communities as the best food for suckling infants. For non breast-fed infants, bovine milk-based formulas have been regarded as a suitable substitute. However, human milk is markedly different from bovine milk in protein amount and composition. In addition, the amino acid composition of human and bovine caseins differ substantially. The predominant amount of casein in bovine milk is Æ-casein which is almost absent in human milk (Kunz and Lönnerdal, 1989). The major subclass of casein in human milk is â-casein and there is a 10-residue shift in amino acid alignment between bovine and human â-caseins (Greenberg *et al.*, 1984). Bovine â-casein is completely phosphorylated (5 phosphates) and forms large micelles. Human â-casein has a variable phosphorylation pattern within the first 10 amino acid residues at the N-terminal end and can donate divalent cations more easily. It also forms smaller and softer micellular curds which may be easier to digest.

In the early 1900s, physicians found that proteins in cow's milk were responsible for gastrointestinal disease and even digestive collapse of newborn infants fed a cow milk-based diet (Schlossmann and Moro, 1903). The allergenicity of cow's milk for the human infant appears to be caused predominantly by â-casein (Salvilathi and Kuitunen, 1992). The sensitivity of infants to bovine casein is remarkable. Reports of infants experiencing anaphylactic shock from the breast milk of mothers who drink cow's milk are not uncommon (Lifschitz *et al.*, 1988). The infants' adverse reactions to breast milk ceased when the mother stopped consuming cow's milk, indicating that intact casein or another bovine milk protein or possibly antigenic fragments were absorbed through the mother's gut and released into breast milk in sufficient quantities to elicit anaphylaxis in the infant. Differences in amino acid sequence are considered to be the source of infant allergies (Miller *et al.*, 1990). Despite the immunological problems with cow's milk and cow's milk-based formulas, they have become the primary source of infant nutrition in the Western world due to their availability and convenience. Although breast-feeding may be the best way to provide newborns with the benefits and protection of human milk-based nutrition, it is not always physiologically possible or culturally permissible. To construct plant-based infant formulas that resemble the nutrition of human milk, we describe the transformation of potato plants (*S. tuberosum*, cv. Bintje), with cDNA encoding human â-casein. The plant-produced â-casein is identical in amino acid sequence to the human â-casein and can be used in infant formulas to avoid the immunological and physiological barriers presented by ingestion of cow's milk â-casein. In this work, we provide the successful first step in development of a food plant-based human milk protein source for infant formula to provide newborn infants with the benefits of human milk. In the future, introduction of additional human milk proteins such as, lactoferrin and lysozyme into food plants already containing human casein may provide a more complete human milk for optimum growth benefits and protection against infant diseases.

Materials and methods

Cloning of Hbca cDNA in plant expression vector pPCV701FM4

The human â-casein (Hbca) cDNA was originally subcloned into the *Sma*I site of the pGEM-3Z, resulting in recombinant plasmid pG3hâcas.4 (Menon and Ham, 1989). The â-casein cDNA fragment was excised from the recombinant plasmid pG3hâcas.4 by double digestion with *Xba*I and *Sac*I in the same buffer. The DNA fragment was inserted into the multiple cloning site of plant expression vector pPCV701FM4 between *Xba*I and *Sac*I sites. The resulting plasmid was named pPCV701FM4-Hbca (Fig. 1). Plant expression vector pPCV701FM4 was constructed by addition of a multiple cloning site (*Bgl*II, *Xba*I, *Sma*I, *Spe*I, *Sac*I, *Srf*I, *Bam*HI) to the *Bam*HI site of pPCV701 LuxFM1 (Koncz *et al.*, 1988; Jiang *et al.*, 1992). To increase the number of cloning sites and the addition of endonuclease *Srf*I for PCR cloning, a polylinker (*Bgl*II, *Xba*I, *Sma*I, *Spe*I, *Sac*I, *Srf*I, *Bam*HI) was designed. Two partially complementary oligonucleotides of the polylinker DNA sequences were synthesized with the 394 DNA/RNA synthesizer (Applied Biosystems, Inc.). After phosphorylation and annealing, the double stranded synthetic polylinker was ligated into the *Bam*HI cut and dephosphorylated pPCV701FM1 vector. In order to retain a single *Bam*HI site in the multiple cloning site, the polylinker was designed to have *Bgl*II at one end, thereby restoring one of the *Bam*HI sites during ligation.

Plasmid pPCVl701FM4-Hbca was introduced into competent *E. coli* strain HB101 by electroporation methods as described by Dower *et al.* (1987). Plasmid DNA was isolated from ampicillin resistant (Ap^r) *E. coli* colonies. The recombinant plasmid containing the *Hbca* cDNA insert was identified by DNA fragment pattern analysis following restriction endonuclease digestion and agarose gel electrophoresis of the DNA fragments. The DNA sequence of the *Hbca* cDNA was verified by PRIM™ ready reaction DyeDeoxy™ terminator cycle sequencing (Applied Biosystems, Inc.) with four primers designed to cover the entire *Hbca* coding region. Purified plasmid DNA of pPCV701FM4-Hbca, harbouring the correct *Hbca* cDNA sequence, was introduced into *A. tumefaciens* strain GV3101 pMP90RK (Koncz *et al.*, 1987) by electroporation using a Bio-Rad Gene Pulser electroporation apparatus according to the manufacturer's instructions. Plasmid DNA from several carbenicilin-resistant ($Carb^r$) colonies was isolated and reintroduced

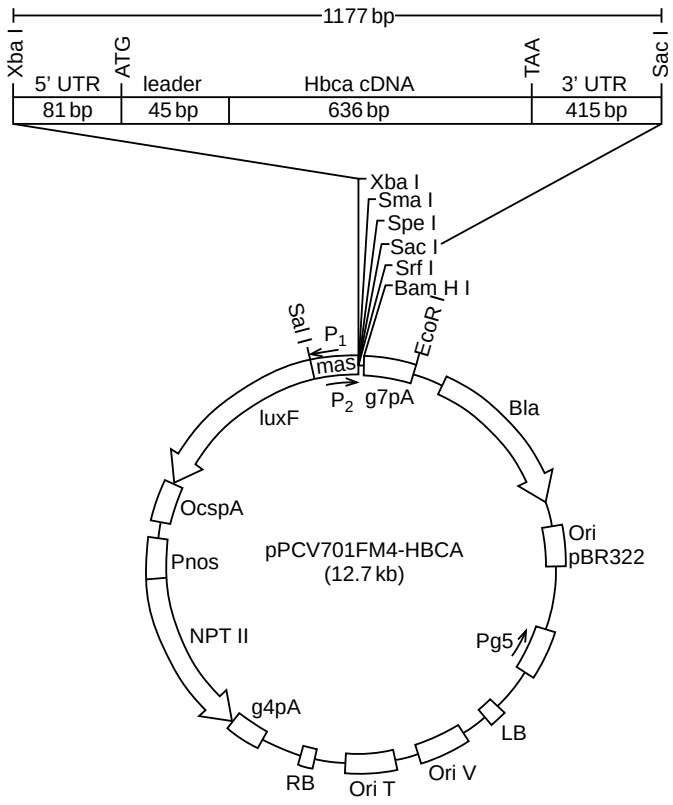

Fig. 1. Structure of the plant expression vector pPCV701FM4-Hbca. The 1177 bp *Hbca* insert from pG3hâcas was subcloned into the *Xba*I and *Sac*I sites of the pPCV701 FM4, to give the plasmid pPCV701FM4-Hbca. The recombinant plasmid carries the left and right 25 bp direct repeats (LB and RB) of the transferred DNA (T-DNA), which borders the region incorporated into the plant genome. Human â-casein cDNA was placed downstream of a 430 bp DNA fragment containing the P2 promoter of the mannopine synthase gene (*mas*) and was followed by the polyadenylation sequence of the T_L-DNA gene 7 (g7pA). The bacterial luciferase fusion gene (*lux*F) was driven by the mas P1 promoter and is terminated by the polyadenylation sequences of the octopine synthase gene (OcspA). Arrows indicate the direction of transcription. Restriction endonuclease cleavage sites are indicated. pNOS, promoter of nopaline synthase gene; NPTII, neomycin phosphotransferase gene from transposon Tn5; g4pA, polyadenylation sequences of the T_L-DNA gene 4; OriV, OriT, replication and transfer origin sequences derived from plasmid pRK2; Pg5, promoter of T_L-DNA gene 5; Ori pBR322, replication origin of pBR322, Bla, â-lactamase gene conferring resistance to ampicillin.

into *E. coli* HB101 for further confirmation of the plasmid DNA sequence by restriction mapping, since recombination can occur in *Agrobacterium* and it is difficult to isolate plasmid DNA from that species. A colony of *A. tumefaciens* containing pPCV701FM4-Hbca DNA was selected for the plant transformation experiments.

Plant cell transformation

Leaf explants of *S. tuberosum* cv. Bintje were cocultivated with *A. tumefaciens* strain GV3101 pMP90RK harboring plasmid pPCV701FM4-Hbca on solid Murashige and Skoog plant growth medium containing 0.2% gelrite and plant hormones (0.1 mg l^{-1} naphthalene acetic acid (NAA) and 1 mg l^{-1} *trans* zeatin (Sigma)), as well as 100 mg l^{-1} kanamycin. After 3 days' incubation at 29 8C, the leaf explants were transferred to the same culture medium containing 400 mg l^{-1} claforan for counterselection against *A. tumefaciens*. The explants were incubated in the light room at 12 ìE and 12 h photoperiod for 3–4 weeks to permit shoot formation. Leaf explants containing friable callus tissues were transferred to growth medium minus NAA for an additional 2–3 weeks to facilitate shoot formation. Regenerated shoots were rooted in the medium without plant growth hormones. Roots appeared on the transgenic shoots after 2–3-week incubation and the regenerated plants were transferred to soil and grown to maturity in a greenhouse. Leaves from transformed plants were subjected to low light image analysis (Hamamatsu Argus-100 intensified video camera) to detect light emission from the plant tissue indicating the presence of the luciferase marker gene (Langridge *et al.*, 1989). Light emitting transgenic plants were selected for further genetic and biochemical analysis.

The presence of the *Hbca* gene in transformed plant tissues was determined using the polymerase chain reaction with a forward primer, 59-ACCAATACATTA-CACTAGCATCTG-39, specific for the *mas* P2 promoter and a reverse primer, 59-GACTGAGTGCGATATTATGT-GTAATAC-39, specific for the polyadenylation region of the T_L DNA gene 7 (g7pA). Plant genome DNA was isolated from leaf tissue of transgenic potato plants using a plant DNA isolation kit (Boehringer-Mannheim). DNA samples were quantified by spectrophotometric measurement of UV absorbance at 260 nm. The polymerase chain reaction was carried out in a 100 ìl volume containing 1 3 *Taq* DNA polymerase buffer without $MgCl_2$, 3 mM $MgCl_2$, 200 ìM dNTP, 0.5 ìM of each primer, 2.5 units of *Taq* DNA polymerase, and 500 ng genomic DNA. The mixture was initially denatured at 95 8C for 7 min and followed by 30 cycles of 45 s at 95 8C, 1 min for annealing at 60 8C, and 2 min for extension at 72 8C. The extension reaction was continued at 72 8C for 10 min before cooling the preparation to 4 8C. The expected PCR product is the entire *Hbca* cDNA. The presence and approximate copy number of *Hbca* cDNA were detected by Southern blot analysis (Southern, 1975). Approximately 10 ìg of genomic DNA was digested with *Bam*HI. The DNA fragments were separated by electrophoresis on 0.8% agarose gels, and transferred to a positively charged nylon membrane (Boehringer-Mannheim). Transgenic potato genomic DNA fragments were hybridized with a DIG-PCR labelled *Hbca* probe

synthesized by incorporation of Digoxigenin-11-dUTP during PCR with primers specific for *Hbca* cDNA (5′-forward primer, 5′-GGTGGATCCTGCTCTTATGAGG-GAGACCAT-3′, and 3′-reverse primer, 5′-TTAACTTT-GAGCTCTTCTTAGAC-3′). DNA hybridization was carried out overnight at 68 °C in a solution containing 5 × SSC, 1.0% (w/v) blocking reagent, 0.1% *N*-lauroylsarcosine and 0.2% SDS. The DIG-labelled casein DNA probe was detected by incubating the hybridized membrane in the chemiluminescent substrate Lumi-Phos 530 sandwiched between two sheets of clear acetate for 30 min at 37 °C followed by a 10 min exposure of the membrane on RX Fuji X-ray film.

Detection of Hbca mRNA

Total RNA from uninduced leaf tissue of transformed plants was isolated using an RNeasy plant kit (Qiagen Inc.). The presence of *Hbca* messenger RNA was detected in uninduced transgenic plant leaf tissues by reverse transcription and polymerase chain reaction (RT-PCR) analysis methods. The first strand of cDNA was synthesized from the *Hbca* mRNA by AMV reverse transcriptase with the 3′-reverse primer (5′-TTAACTTT-GAGCTCTTCTTAGAC-3′) of Hbca. Plant total RNA (2 µg) from each sample was denatured at 65 °C for 5 min and then added to the reverse transcription mixture containing 1 × AMV transcriptase incubation buffer, 1 mM dNTP, 1 µM *Hbca* 3′-reverse primer, 1 unit per µl RNase inhibitor, and 25 units of AMV transcriptase. The reaction mixture was incubated at 25 °C for 10 min to anneal the primers to the RNA templates, followed by incubation at 42 °C for 60 min to reverse-transcribe the RNA into cDNA. At end of the reaction the DNA/RNA hybrids were denatured at 95 °C for 5 min and cooled to 5 °C for 5 min. The resulting first strand of cDNA was subsequently used as template for PCR synthesis of the second cDNA strand with the 5′-forward primer (5′-GGTGGATCCTGCTCTTATGAGGGAGACCAT-3′), and the same Hbca 3′-reverse primer. The *Hbca* cDNA was amplified in 100 µl of solution containing 5 µl of the reverse transcription mixture, 200 µM dNTP, 1 × *Taq* DNA polymerase buffer with 1.5 mM $MgCl_2$, 0.5 µM each of the *Hbca* primers, and 2 units of *Taq* DNA polymerase. The mixture was initially denatured at 95 °C for 3 min and followed by 30 cycles of 95 °C for 45 s, 55 °C for 1 min and 72 °C for 1 min with a final incubation at 72 °C for 10 min. The RT-PCR product was separated by electrophoresis on a 1.2% agarose gel. The molecular weight of the RT-PCR product corresponded to the full-length coding region of the *Hbca* gene.

Immunoblot detection of Hbca protein

Prior to analysis, leaf and microtuber tissues from transgenic potato plants grown in sterile glass jars in a light room on a 12 h photoperiod were wounded by cutting repeatedly with a sterile razor blade and incubated for 3 days on solid culture medium containing 10^{-3} molar NAA to stimulate the *mas* promoters (Langridge *et al.*, 1989). Total soluble protein was extracted from the induced potato leaf and tuber tissues by homogenization with a mortar and pestle on ice in 4 °C extraction buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 1 mM PMSF and 1% Triton X-100.

Approximately 200 mg of leaf tissue was homogenized in 600 µl of extraction buffer and 200 mg of tuber tissue in 300 µl of extraction buffer. The homogenates were centrifuged in a GS-15R Beckman tabletop centrifuge at maximum speed (15 000 × **g**) for 15 min at 4 °C. Total soluble protein concentration in the supernatant was measured using a BioRad Bradford protein assay kit. Approximately 150 µg total protein from each sample was denatured by boiling for 5 min in sample loading buffer. The boiled samples were separated by electrophoresis on 12% SDS-PAGE, and the protein bands were transferred from the gel by electroblotting onto a PVDF membrane. The damp membrane was blocked with 1% BSA and 20% foetal calf serum in TBST buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.05% Tween 20) for at least 1 h. The blocked membrane was incubated for 1 h in TBST solution containing a 1:750 dilution of rabbit anti-β-casein, affinity-purified polyclonal antibody. The anti-Hbca primary antibody/Hbca antigen complex was conjugated with a secondary antibody by incubation of the membrane in a 1:10 000. TBST dilution of a mouse anti-rabbit IgG alkaline phosphatase conjugate (Sigma A-2556). Following binding of the secondary antibody, the membrane was washed three times in TBST solution to remove unbound secondary antibody. Colorimetric detection of the recombinant protein was accomplished by incubating the membrane in BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) substrate solution for 10–30 min at room temperature.

Results

Vector construction and plant transformation

The *Hbca* cDNA was inserted into the multiple cloning site of the pPCV701FM4 between *Xba*I and *Sac*I to give plasmid pPCV701FM4-Hbca. In this construct, the *Hbca* gene was placed under transcriptional control of the *mas* P2 promoter (Velten *et al.*, 1984). The bacterial luciferase transcriptional fusion gene (Escher *et al.*, 1989) was driven by the P1 site of the *mas* P1, P2 dual promoter. The bacterial luciferase transcriptional fusion gene has been used as a reporter gene for the screening of transgenic plants by low-light video analysis (Langridge *et al.*, 1991). In addition, construct pPCV701FM4-Hbca contains the neomycin phosphotransferase II (NPTII) gene

for selection of transformed plants on kanamycin-containing growth medium.

Potato leaf explants (*S. tuberosum* cv. Bintje) excised from sterile plants were transformed by *A. tumefaciens*-mediated transformation methods. Leaf explants were infected by *A. tumefaciens*, strain GV3101 pMP90RK, harbouring the construct pPCV701FM4-Hbca and cultured on selective medium. The kanamycin-resistant putative transformants were screened for the presence of the detectable marker gene by low-light video image analysis, and six independent potato transformants were obtained which had strong light emission in comparison with uninfected control plants. In addition to potato, transformation of *N. tobacum* cv. Samsun with the pPCV701FM4-Hbca was also attempted. Ten kanamycin-resistant tobacco plants were regenerated. Tissues from eight of the ten plants exhibited bioluminescence detected by low light video image analysis. Due to our emphasis on nutrition, however, further analyses of *Hbca* gene expression were carried out only on transgenic potato plants.

Detection of Hbca DNA in potato transformants

The presence of *Hbca* cDNA in the genomic DNA isolated from regenerated potato transformants was detected by PCR amplification of *Hbca* inserts with 5′ and 3′ flanking primers. The expected PCR product was a 1309 bp fragment and was detected in all six transgenic potato plants (Fig. 2A, lane 4–9). No DNA product was detected in untransformed control plant DNA (Fig. 2A, lane 3). The same fragment was amplified using plasmid pPCV701FM4-Hbca as a template (Fig. 2A, lane 1). The PCR-generated *Hbca* fragment was confirmed further by restriction endonuclease mapping. Based on DNA sequence analysis, the enzyme *Bgl*II cuts the *Hbca* cDNA once and produces two fragments from the PCR-generated product with molecular sizes of 769 bp and 540 bp, respectively. These two DNA fragments were produced after *Bgl*II digestion of all six lines of transgenic potatoes (Fig. 2B, lane 2–7). From 1 to 3 copies of *Hbca* gene were detected in the six transgenic potato plants by Southern hybridization methods (data not shown).

Human â-casein mRNA was detected in uninduced leaf tissue of all six transgenic potato plants using RT-PCR methods. In the reverse transcription reaction, a 3′ reverse primer was designed to synthesize the first *Hbca* cDNA strand starting from the stop codon of *Hbca* cDNA. In combination with a 5′ forward primer which is located at the start codon of *Hbca* cDNA, the full length coding region of the *Hbca* gene was amplified directly from the reaction mixture of RT from all six transgenic potato plants (Fig. 3, lane 3–8). No RT-PCR product was observed from RNA isolated from untransformed potato plants (Fig. 3, lane 2). The *Hbca* cDNA coding region was amplified when pPCV701FM4-Hbca plasmid DNA was used as the template (Fig. 3, lane 1). To eliminate the possibility of DNA contamination in the plant total RNA preparation, the RNA sample was used as a

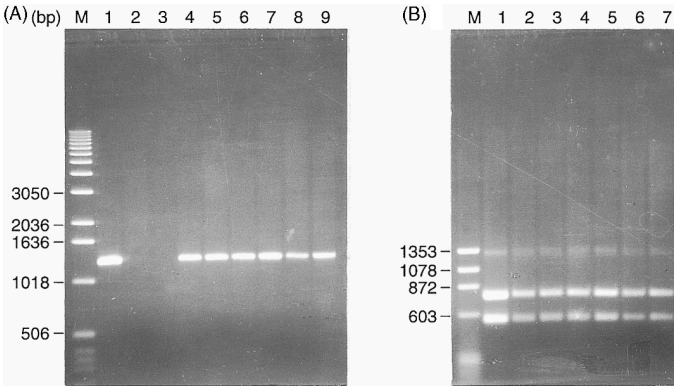

Fig. 2. PCR detection of human â-casein cDNA in transgenic plants (A) and *Bgl*II digestion of PCR products (B). In Fig. 2A, lane M is the 1 kb DNA ladder; lane 1, PCR product of plasmid pPCVFM4-Hbca; lane 2, H_2O control; lane 3, untransformed plant. Lane 5–9 are PCR products from transgenic potato plant, Bin-hbc 1, Bin-hbc 2, Bin-hbc 3, Bin-hbc 4, Bin-hbc 5 and Bin-hbc 6. In Fig. 2B lane M is ÖF174RF *Hae*III fragments and lane 1 is *Bgl*II digestion pattern of PCR product from plasmid pPCVFM4-Hbca and lane 2–7 are patterns from the six individual transgenic potato plants.

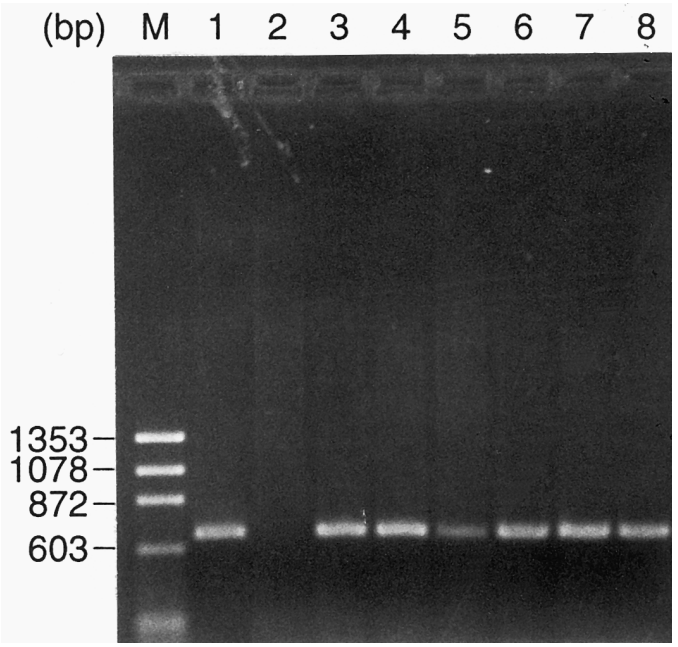

Fig. 3. RT-PCR analysis of Hbca RNA from transgenic potato plants. Lane 1, RT-PCR product from plasmid pPCVFM4-Hbca; lane 2, untransformed plant; lane 3–7, transgenic potato Bin-hbca 2, Bin-hbca 3, Bin-hbca 4, Bin-hbca 5, Bin-hbca 6; and lane M, ÖX174RF *Hae*III fragments.

template for PCR. No amplified DNA products were detected (data not shown).

Human â-casein gene expression

Recombinant â-casein protein was detected in leaf tissue homogenates by immunoblot methods using an affinity-purified rabbit polyclonal antibody specific for human â-casein. During electrophoresis, the plant-produced â-casein migrated as a single band in the SDS-PAGE gel with a molecular weight of approximately 30 kDa, which was about 1–1.5 kDa smaller than the phosphorylated human â-casein 2P isoform (Fig. 4, lane 5, 7, 9 and 11). No protein band was observed in untransformed plant homogenates (Fig. 4, lane 3). In pPCV701FM4-Hbca, the mannopine synthase gene dual P1, P2 promoters are up-regulated predominantly by the plant growth hormone auxin (Langridge *et al.*, 1989). In addition, *mas* promoter activity can be induced further by wounding the plant leaf or tuber tissue 24–48 h prior to assay. The *mas* promoter activity in uninduced transgenic plant tissues was barely detectable, based on low-light image analysis of luciferase activity (Langridge *et al.*, 1989). In this experiment, uninduced tissue samples of the transgenic plants did not show a protein band on immunoblots at the molecular weight of Hbca protein (Fig. 4, lanes 4, 6, 8 and 10) as compared with the wounded and auxin induced leaf samples from the same plants (Fig. 4, lanes 5, 7, 9 and 11). No corresponding band was detected in either the induced or uninduced leaf tissue samples from untransformed plants (Fig. 4, lanes 2 and 3). In comparison with human â-casein 2P standard on immunoblots, the amount of recombinant â-casein in wounded and auxin-induced leaf tissues was estimated to be approximately 100 ìg â-casein per gram of soluble leaf protein. This value represents approximately 0.01% of total soluble potato plant protein. The expression of *Hbca* in potato tuber tissue determined by immunoblot analysis was low in comparison with luciferase enzyme activity detected by bioluminescence assay. There was a barely visible antibody-reacting band in tubers of transgenic plants in response to auxin induction. However, â-casein was detected in potato tuber slices after 5 days auxin induction by immunoprecipitation methods. The recombinant â-casein was immunologically precipated from the total soluble protein extracts with an anti-â-casein antibody using a protein A immunoprecipation kit from Boehringer-Mannheim. A protein band of identical mobility to the recombinant â-casein from leaf total soluble protein extracts was observed on immunoblots from the immunoprecipated sample of tuber tissue (data not shown). In addition, the phosphoprotein fraction of potato soluble proteins was obtained by glacial acetic acid and ethanol precipitation at pH 4.6 from leaf homogenates of transgenic and untransformed plants (Linebaugh and Rillema, 1984). A protein band with the same mobility as recombinant â-casein from leaves was obtained on immunoblots from the phosphoprotein fraction of transgenic plants but not from untransformed control plants (data not shown).

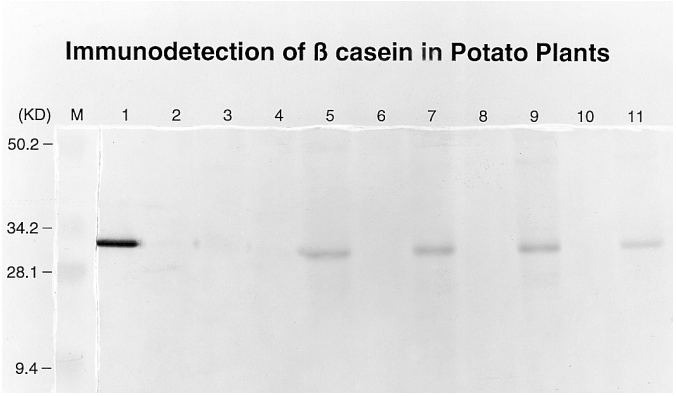

Fig. 4. Immunodetection of â-casein protein from leaf extract of the transgenic potato plants. Total soluble protein extract (150 ìg/lane) from potato leaves was fractionated by SDS-PAGE, blotted onto a PVDF membrane, and probed with a rabbit anti-â-casein polyclonal antibody. Lane 1 is the 2P isoform of human â-casein isolated from human milk (50 ng). The remaining lanes contain proteins isolated from leaf tissue in which the *mas* promoters were either not induced or were induced by incubation on culture medium containing auxin (10^{-4} M). Lane 2 and 3 contain proteins from uninduced and induced untransformed plants, respectively. Lane 4, 6, 8, and 10, were loaded with proteins from the uninduced leaf tissue of four selected transgenic plants, Bin-hbca 1, Bin-hbca 2, Bin-hbca 3, and Bin-hbca 4. Lane 5, 7, 9, and 11 contain proteins from the induced leaf tissue of the above-mentioned four transgenic plants.

Discussion

We have engineered transgenic *S. tuberosum* cv. Bintje potato plants to express the human cDNA encoding â-casein, a major calcium binding protein from human milk. The recombinant protein was detected by immunoblot analysis in both leaf and tuber tissue using an affinity-purified polyclonal antibody specific for human â-casein. The *Hbca* gene product was detected in total soluble protein extracts of leaves. However, expression of *Hbca* cDNA in auxin-stimulated, wounded tuber tissues was easily detected only after immunoprecipitation. We have observed a similar result in *S. tuberosum* potato plants transformed with the human insulin gene under control of the *mas* P2 promoter (unpublished data, 1996). This result suggests that the *mas* P2 promoter may be less active in tubers than in leaf tissue. The possibility of proteolytic degradation of â-casein protein in tuber tissues can not be excluded at this time.

Based on the results of immunoblot experiments, plant-produced â-casein protein appears to be a single peptide

approximately 1.0–1.5 kDa smaller than the human â-casein 2P isoform (Fig. 4). Since full-length *Hbca* mRNA was detected by RT-PCR (Fig. 3), the apparent reduction in size of the plant â-casein protein may be due to differences in post-translational modifications between plants and animal cells, such as phosphorylation and glycosylation. During phosphorylation of the human â-casein protein, attachment of the first phosphate group adds approximate 1.5 kDa to the apparent molecular weight, possibly by changing the conformation of the protein. However, when additional phosphates are added to the molecule, the molecular weight is not significantly increased (Hansson *et al.*, 1993). Human â-casein from milk is phosphorylated at the N-terminal end of the peptide. Depending on the phosphorylation state, from none to five phosphates can be attached to serine and threonine residues within the first 10 amino acids from the N-terminal end of the protein. We have attempted to isolate phosphoproteins from transgenic leaf protein homogenates. The results of these experiments indicate that â-casein synthesized in the plant may be less phosphorylated than breast milk â-casein as the molecular mass is smaller than phosphorylated â-casein from human milk. There is no indication in the literature that â-casein from human milk is glycosylated. Preliminary N-terminal sequence analysis of plant synthesised â-casein indicate that the N-terminus may be blocked. The possibility of â-casein serine residue glycosylation in the plant could increase the net negative charge on the protein which would result in increased protein mobility during electrophoresis. This increase in electrophoretic mobility may account for the observed size reduction of potato â-casein in comparison with breast milk â-casein. Further, correct processing of the leader peptide in the plant, in addition to phosphorylation of potato synthesized human â-casein could also result in similar reduction of potato â-casein size. Human â-casein cDNA encodes a 15 amino acid leader sequence with an approximate molecular weight of 1.5 kDa. The fact that â-casein synthesized in the plant is smaller than â-casein from human milk suggests that the 15 amino acid leader sequence has been removed in potato. Immuno-affinity column purification experiments are in progress to isolate larger amounts of plant synthesized human â-casein for N-terminal amino acid sequence and phosphorylation pattern analyses.

To determine the maximum amount of human â-casein produced in transgenic potato, callus tissue was regenerated from transformed leaf and tuber tissue cultured on a medium containing 10^{-3}–10^{-4} M auxin. Immunoblot analysis of this callus tissue not yet performed, may indicate amounts of human â-casein protein approaching 0.5% of total soluble protein, similar to results recently obtained in transgenic potato callus producing cholera B toxin protein (data not presented, 1996). Based on previous immunoblot experiments, the amount of â-casein presently synthesized from the *mas* P2 promoter in transgenic potato tissues, is approximately 0.01% of total soluble protein. To obtain increased levels of â-casein expression in tuber tissues additional cloning and potato transformation experiments are underway using plant expression vectors containing the tandem cauliflower mosaic virus 35S promoter or the potato patatin promoter fused to *Hbca* cDNA. In potato plants, the patatin promoter produces up to 10% of total patatin synthesis as recombinant protein (Spychalla *et al.*, 1994). Since patatin makes up approximately 20% of total soluble potato protein, the amounts of recombinant human â-casein can be as high as 2%. If tuber soluble protein content is approximately 23 mg per gram fresh weight (Pennington, 1993); and if about 33 400 pounds of potatoes are produced per acre according to US Department of Commerce 1995 estimates, approximately 6.4 kg of human â-casein can be produced per acre. Based on a value of 0.9 g â-casein per litre of human milk (Miller *et al.*, 1990), the amount of â-casein produced per acre of potatoes equals about 7076 litres of human milk. It is clear that utilizing light energy, large amounts of â-casein can be inexpensively synthesized in transgenic food plants. The cloning and expression of human â-casein DNA in potato opens the way for the biosynthesis of additional human milk proteins such as lactoferrin, lysozyme and Æ-lactalbumin in food plants. The reconstitution of human milk in edible plants will ultimately lead to the development of infant formulas and solid baby foods with increased nutritional and therapeutic potential as well as increased protection of neonates against the development of food allergies and autoimmune diseases.

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