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Synthesis and self-assembly of a functional monoclonal antibody in transgenic *Nicotiana tabacum*

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Abstract

Immunoglobulin light and heavy chains are synthesized in mammalian cells as precursors containing a signal peptide. Processing and assembling result in formation of active antibodies. Chimeric genes have been made containing the coding sequence of the barley α-amylase signal peptide which has been fused to cDNAs coding for either the mature light or the mature heavy chain of a monoclonal antibody. A plasmid was constructed linking both chimeric genes under the control of plant active promoters in an expression cassette. This DNA fragment was stably integrated into the genome of *Nicotiana tabacum* by *Agrobacterium tumefaciens* mediated gene transfer. Synthesis of light and heavy chains and assembly to antibodies was detected in transgenic tobacco tissue using specific secondary antibodies. By electron microscopic immunogold labeling, the presence of assembled antibody could be detected within the endoplasmic reticulum. Affinity chromatography indicated biological activity of the assembled immunoglobulin produced in plant cells. Unexpectedly, a significant amount of assembled antibodies was found within chloroplasts.

Introduction

Expression of light and heavy chains of monoclonal antibodies in non-lymphoid tissues has become an important area of research during the past years. In native lymphoid cells, immunoglobulin light and heavy chains are synthesized as precursor proteins. The signal peptides direct insertion of the nascent chains into the lumen of the endoplasmic reticulum (ER). Inside the ER, a heavy chain binding protein (BiP) interacting with noncovalently associated immunoglobulin heavy chains mediates assembly of immunoglobulin light and heavy chains [4, 17, 20]. Recently a relationship of BiP to other proteins regulating transport of secretory and membrane proteins (GRP 78 and HSP 70) was reported. These proteins seem to have a general function in stress management [12, 18, 21, 26].

In *E. coli*, expression of light and heavy chains of monoclonal antibodies lacking the natural signal peptides did not result in the production of

functional antibodies within the transformed bacterial cells. Only *in vitro* reconstitution of bacterial extracts or purified fractions containing both chains resulted in the recovery of antigen binding activity [6, 9]. This was ascribed to the highly reducing intracellular environment inhibiting disulfide-bond formation or the lack of a special helper protein [9] or simply to low level expression [5]. Expression of a native light chain precursor gene produced a secreted immunoglobulin chain [46]. Recently, fusion of both intact or truncated light and heavy chains to bacterial signal peptides and expression of these chimeric genes in *E. coli* was successful. Protein engineering allowed the assembly of secreted functional chimeric antibodies or fragments linked by disulfide bonds [1, 35].

The immunoglobulin chains expressed by Boss *et al.* [5] in *E. coli* were also expressed in yeast cells but in this case as native precursor proteins [43]. Synthesis, processing and secretion of light and heavy chain, glycosylation of heavy chain and detection of functional antibodies in extracts from cells co-expressing both chains was demonstrated. Yet, the efficiency of assembly was low. By fusion of chimeric immunoglobulin chains to the yeast invertase signal peptide Horwitz *et al.* [22] achieved secretion of properly folded and assembled chimeric antibody and F_{ab}-fragment from yeast cells.

We have constructed chimeric genes consisting of the mature light and heavy chain genes, derived from cDNA clones of the B 1-8 antibody [6, 7] and the barley aleurone α-amylase signal peptide coding sequence [13, 14]. This signal peptide has already been used successfully to direct transport of bacteriophage T4 lysozyme from transgenic tobacco cells to the intercellular spaces [15, 23]. Thus, initiation of the secretory pathway was also expected for the chimeric immunoglobulin chains in transgenic plant tissue.

Transgenic tobacco tissue synthesizing both immunoglobulin chains was shown to produce functionally active B 1-8 antibody by using specific interaction with its antigen. Subcellular localization indicates assembly of the antibody in the endoplasmic reticulum. Synthesis and assembly of a complex foreign protein to a biologically active molecule inside transgenic plant cells is possible by fusing the individual chains to a plant signal peptide.

Materials and methods

All cloning and DNA manipulations were performed according to Maniatis *et al.* [30].

Plasmids, bacterial strains, and media

Plasmids and bacterial strains are listed in Table 1. pAB λ 1-15 and pAB μ-11 were gifts from A. Radbruch; pLC 1-4, pHC 1-3, pHC 3-19 and pLGV 2385 *Bgl*→*Hin*d from M. Stieger.

Recombinations, transformations and conjugations were carried out as described elsewhere [15].

Construction of chimeric signal peptide light- and heavy-chain genes

The DNA sequence coding for the signal peptide of α-amylase from barley aleurone layer (*Clone E* [33]) was fused to the cDNA fragment coding for the mature light chain of B 1-8 antibody [8] (Fig. 1b). The signal peptide coding fragment was excised from pSR 1-1, a pUC 9 plasmid containing the *Clone E* α-amylase cDNA of Rogers and Milliman [14]. A synthetic oligonucleotide providing a 5′ *Hin*d III site was ligated under conservation of the *Nco* I site which overlaps with the ATG initition codon of the signal peptide. The mature light chain coding sequence was excised from plasmid pLGV 2385 Lc. The isolated signal peptide coding sequence was fused at its 3′ end to a synthetic oligonucleotide providing the 3′ nucleotides of the signal peptide coding sequence and the 5′ nucleotides of the light chain gene. The complete chimeric gene was ligated in the correct orientation into the selection-expression vector pAP 2034 [41] containing the T_R-1′ 2′ dual pro-

Table 1. Plasmids and bacterial strains.

Bacterial strains	Plasmids	References
E. coli		
DH 1		Low [29]
BMH 71-18		Yanish-Perron *et al.* [44]
GJ 23		Van Haute *et al.* [40]
Agrobacterium		
C58 C1		Zambryski *et al.* [45]
	pAB λ 1-15	Bothwell *et al.* [8]
	pAB μ-11	Bothwell *et al.* [7]
	pLC 1-4	Stieger [38]
	pHC 1-3	Stieger [38]
	pHC 3-19	Stieger [38]
	pLGV 2385 Lc	Stieger [38]
	pLGV 2385 *Bgl*→ *Hin*d	Stieger [38]
	Clone E	Rogers and Milliman [33]
	pAP 2034	Velten and Schell [41]
	pUC 9	Vieira and Messing [42]
	pBR 322	Bolivar *et al.* [5]
	pGV 3850	Zambryski *et al.* [45]
	pSR 1-1	Düring *et al.* [15]

moter and the gene 7 polyadenylation site (derived from the T-DNA of *Agrobacterium tumefaciens*) (pSR 1-4).

An analogous construction was built with the mature heavy chain coding sequence (Fig. 1c). The signal peptide coding sequence was again prepared from plasmid pSR 1-1 but fused to another synthetic oligonucleotide at the 3′ end comprising the missing 3′ nucleotides of the signal peptide coding sequence and the 5′ part of the gene coding for the mature heavy chain.

For integration into the plant genome a transformation vector containing both chimeric genes under control of plant active regulatory elements within a short distance was constructed. This final transformation vector contains under control of the pT_R dual promoter the NPT II gene as a selectable marker (in the 2′ position) and the chimeric light chain gene (in the 1′ position) and under control of the pNOS the chimeric heavy chain gene (Fig. 1a).

This plasmid was introduced into the plant transformation vector pGV 3850 [45] by homologous recombination. Recombinant *Agrobacterium* was tested for correct integration of the plasmid by Southern blotting (modified following Southern *et al.* [37], data not shown).

Plant transformations

Transformations of *Nicotiana tabacum* W38 plants grown in sterile culture were performed as described elsewhere [15].

Demonstration of transformation of regenerated plants

DNA analysis of transformed plants regenerated from the leaf disk infections were done according to Southern [37] and Maniatis *et al.* [30]. NPT-activity tests were performed following the protocols of Reiss *et al.* [31] and Schreier *et al.* [35].

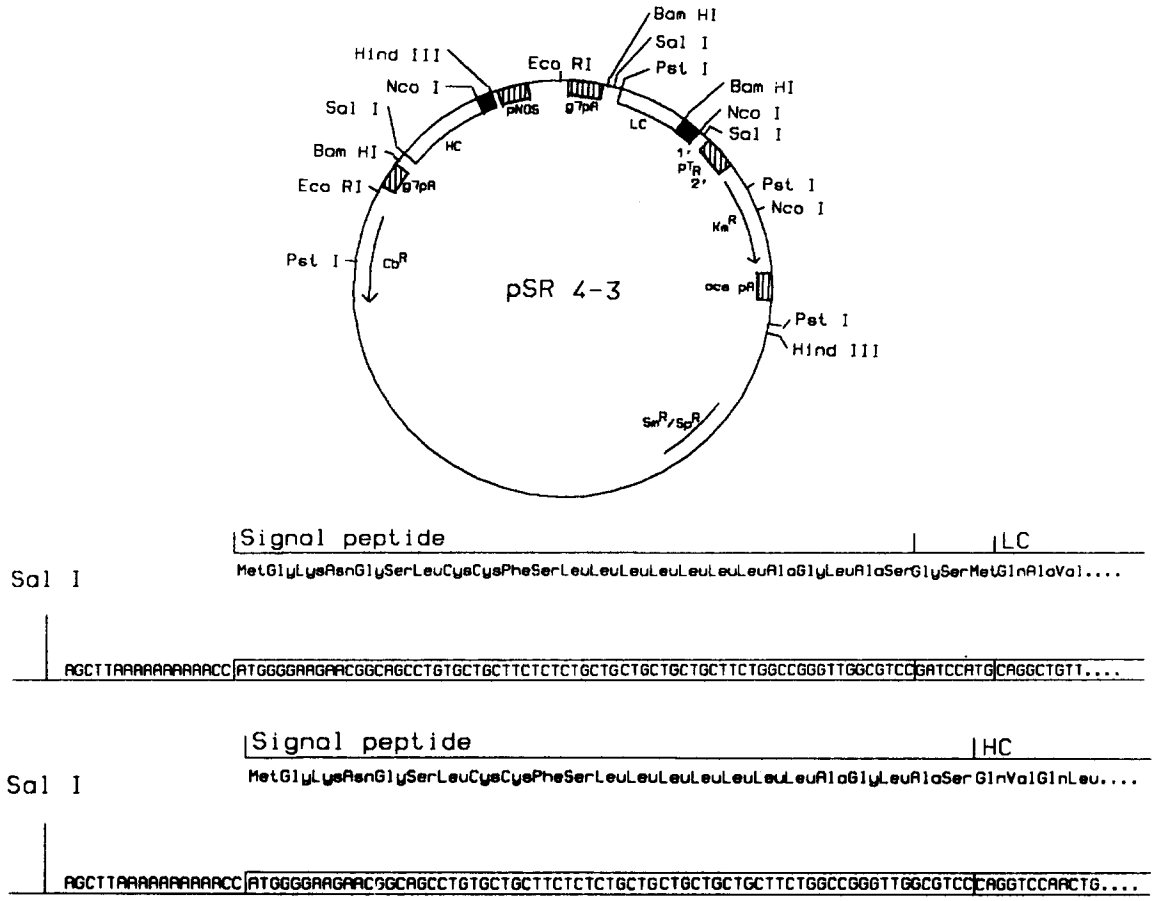

Fig. 1. Construction of chimeric genes consisting of the barley aleurone α-amylase signal peptide coding sequence and the mature B 1-8 light- and heavy-chain cDNAs. Description of the construction in 'Materials and methods'. a (top): Structure of the transformation vector pSR 4-3 containing both chimeric genes under control of plant active regulatory elements. b (middle): Structure of the 5′ part of the chime- signal peptide-light chain gene. c (bottom): Structure of the 5′ part of the chimeric signal peptide-heavy chain gene.

Tissue printing

Tissue printing was done modified following the procedure of Cassab and Varner [10]. Nitrocellulose filters were incubated for 30 min in 0.2 M $CaCl_2$ and air-dried. Immobilon PVDF membranes were wetted in methanol and substituted in water. Tissue was cut and weakly pressed with the cut surface on the prepared membrane for about 30 s. Nitrocellulose filters were dried with warm air. Detection is the same as for western blotting (described below). Pre-absorption of the antibodies used for detection by wild-type protein extract is often essential in order to circumvent cross reactions.

Protein analysis by western blotting and affinity purification of foreign protein from transformed plant tissue

B 1-8 protein, Ls 136, Ac 38, goat anti-mouse IgM and goat anti-λ antibodies as well as NP-Sepharose, L136-Sepharose and NiP-cap were gifts from A. Radbruch and M. Reth.

Protein analysis was carried out using a modi-

fied version of western blotting [39]. Plant tissue was homogenized in SDS-sample buffer supplemented by protease inhibitors (1 μM leupeptin, 1 μM pepstatin, 200 μM PMSF, 100 μM EDTA) and loaded on a discontinuous 15% SDS-polyacrylamide gel according to Laemmli [28]. The separated proteins were transferred to an Immobilon PVDF membrane (Millipore, Bedford, USA) by semi-dry electroblotting [27]. For immunodetection the membrane was blocked with 1% gelatin (BioRad, Richmond, USA) at room temperature to avoid nonspecific adhesion of proteins to the membrane. All antibodies were first preincubated for 10 min with wild-type tobacco protein extract to minimize nonspecific protein-protein interactions in TBS buffer (10 mM Tris-HCl pH 8.0; 150 mM NaCl) including 1% Triton X-100, 0.1% Tween 20, 0.3% gelatin and protease inhibitors. After each incubation the membrane was washed 1 × in TBST (TBS + 0.1% Tween 20), 1 × in TBST + 1 M NaCl and 3 × in TBST (each 10 min).

Antibodies used for detection of individual immunoglobulin chains and assembled antibody are described in Table 2.

Table 2. Antibodies.

Monoclonal antibody	Epitope recognized on B 1-8	Reference
Ls 136	Localized only on λ-light chain	Reth [32]
Ac 38	Localized next to the active site, formed by quarternary structure of light and heavy chain	Reth [32]
Polyclonal antibody	**Used for detection of:**	**References**
Goat anti-λ	Light chain	Southern Biotechnol. Assoc.
Goat anti-IgM	Heavy chain	A. Radbruch
Bio-anti mouse IgG	(second antibody)	Sigma
Bio-anti goat IgG	(second antibody)	Sigma

Proteins were detected by a series of incubations with appropriate antibodies. Anti-IgM, Ls 136 and Ac 38 were followed by a biotinylated secondary antibody and the streptavidin-alkaline phosphatase conjugate from BRL (Bethesda, USA). In the case of biotinylated anti-λ the second antibody was omitted. Colour development became visible within a few minutes to some hours after application of NBT (Nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolylphosphate) in 100 mM Tris-HCl pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$.

For affinity chromatographic purification of the foreign protein from transformed plant tissue the plant material was homogenized in a mortar with a pestle under liquid nitrogen cooling. The homogenate was transferred to a Corex tube and extraction buffer (100 mM PBS) and polyvinylpolypyrrolidone (PVPP) were added. Per gram of tissue 50 mg PVPP and 1 ml 1 × PBS supplemented with protease inhibitors (as described above) were added. As a protective agent against oxidation the solution is made 5 mM ascorbic acid. In order to solubilize all membrane encapsulated proteins 1% Triton X-100 or better 10 mM CHAPS may be added as a detergent. After 15 min extraction was finished by centrifugation of cell debris at 10000 rpm at 4 °C in a Sorvall SS 34 rotor (DuPont, Dreieich, FRG). If a detergent was used for solubilization the supernatant had to be diluted to about 0.1% Triton X-100 or 2 mM CHAPS for affinity chromatography. The supernatant was rotated on a roler at 4 °C for 30–60 min to precipitate oligosaccharides. Subsequently the precipitate was centrifuged at 4000 rpm at 4 °C. Total amount of protein was determined by a Bradford assay (BioRad, Richmond, USA) and equal amounts of total protein were used for transformed and control tissue. The protein extract was incubated with NP-Sepharose (the hapten for isolating the functional B 1-8 antibody) or Ls 136-Sepharose (for isolating the light chain) overnight at 4 °C under slow rotation in a batch assay. By centrifugation (4000 rpm, 4 °C in a Varifuge, Heraeus, Osterode, FRG) the affinity gel was precipitated and the supernatant was discarded. The gel was

transferred to an Eppendorf tube. All the following steps were performed at 4 °C. The gel was washed subsequently 1 × with 1 ml 1 × PBS, 1 × with 1 ml 1 × PBS/1 M NaCl and 3 × with 1 ml 1 × PBS; all solutions were supplemented with protease inhibitors. The specifically bound antibody was eluted 3 × with 200 µl of a 10^{-4} M NIP-cap solution [(5-iodo-4-hydroxy-3-nitrophenyl)acetyl-α-aminocaproic acid] from the NP-Sepharose or 3 × with 200 µl of 0.1 M glycin pH 2.8 and immediately neutralized with 0.1 vol 1 M Tris-HCl pH 8.0 (for the Ls 136-Sepharose). The eluate was concentrated by ultrafiltration, desalted by washing with 1 vol water and total volume was reduced to about 10 µl. The same volume of 2 × SDS-sample buffer was added and the sample loaded onto a western gel and analyzed as described above.

Immunogold labeling

Plant tissue was prepared following the high-pressure freezing method in combination with freeze substitution and low-temperature embedding in Lowicryl HM 20 as described elsewhere [23]. Immunocytochemistry followed the same protocol but with some modifications. In some cases an additional blocking agent (3% newborn calf serum) was introduced in the preincubation step. Antibodies used for immunogold labeling were polyclonal biotinylated anti-λ, monoclonal Ls 136 and biotinylated Ls 136 for detection of the light chain and monoclonal Ac 38 for detection of the assembled B 1-8 antibody. Two systems were used for detection: either direct incubation of the first antibody with colloidal gold-adsorbed Protein A (Auroprobe EM Protein A/G 10, Janssen, Beerse, Belgium) for polyclonal antibodies and colloidal gold-adsorbed goat anti-mouse IgG second antibody (Auroprobe EM Goat anti-mouse IgG/G 10; Janssen, Beerse, Belgium) for monoclonal antibodies or colloidal gold-adsorbed streptavidin (Auropobe EM Streptavidin/G 10, Janssen, Beerse, Belgium) for all biotinylated antibodies. In the case of Ac 38 a biotinylated goat anti-mouse IgG second antibody was used to enter the streptavidin system.

Results

Integration of chimeric immunoglobulin genes into tobacco plants

The chimeric plant signal peptide light- and heavy-chain genes (Fig. 1b and c) were cloned into a single plasmid (pSR 4-3, Fig. 1a). This vector is derived from the plant selection-expression vector pAP 2034 [41]. The chimeric light-chain gene was integrated into the single *Sal* I cloning site next to the 1′ position of the pT_R dual promoter. The chimeric heavy-chain gene was set under the control of the pNOS promoter. Both promoters derive from the *Agrobacterium tumefaciens* T-DNA. The plasmid pSR 4-3 was integrated into the modified Ti-plasmid pGV 3850 [45] by homologous recombination. By leaf disk infection followed by selection on kanamycin the chimeric genes were stably integrated into the genome of *Nicotiana tabacum*. Integrity of the transferred DNA was confirmed by Southern blotting (data not shown). The functionality of the dual promoter in transgenic plant tissues was confirmed by testing for activity of the NPT II gene [31, 35] present in the 2′ position of the dual promoter (data not shown).

Detection of immunoglobulin chains and assembled antibodies by affinity chromatography and immunoblotting

For detection and characterization of the monoclonal antibody B 1-8 a set of different anti-antibodies was used (Table 2). Apart from the polyclonal antibodies interacting specifically with light or heavy chains, several monoclonal anti-idiotypic antibodies are available [32]. Ls 136 recognizes the lambda light chain individually as well as in the assembled antibody. The idiotope is determined only by the light chain. Ac 38 can be used to detect the correct assembly within plant cells because the idiotope is formed by parts both of V_H and V_L domains. The idiotope is not part of the antigen binding site but located closely to it. No interaction of Ac 38 does occur with iso-

lated light or heavy chains. The idiotope is presumably located within the hypervariable regions. All the six hypervariable regions of B 1-8 form a cavity around the NP-binding site.

By 'tissue printing' [10] presence of light and heavy chain as well as the assembly to intact antibodies was indicated using different polyclonal and monoclonal anti-antibodies. Callus tissue derived from leaves of transformed plants was used for analysis because activity of both promoters was shown to be highest in calli (K. Düring, unpublished results). In transformed tissue the blue color of the enzymatic reaction overlaid the light-green color originating from chlorophyll partially fixed to the filters. Using

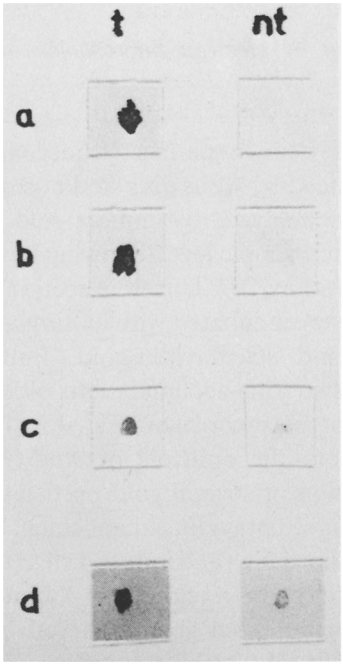

Fig. 2. 'Tissue printing' of callus tissue regenerated from transformed tobacco plants as described in 'Materials and methods'. Weak stainings in the control experiments are due to light-green color originating from chlorophyll in contrast to the dark staining of the blue NBT/BCIP-substrate precipitate in printings from transformed tissue. a. Detection of light chain with biotinylated polyclonal anti-λ antibody. b. Detection of light chain with monoclonal Ls 136 antibody. c. Detection of heavy chain with polyclonal anti-mouse IgM antibody. d. Detection of assembled antibody with monoclonal Ac 38 antibody (t: transformed tissue, nt: wild-type (non-transformed) tissue as control).

polyclonal anti-λ (Fig. 2a) and monoclonal Ls136 (Fig. 2b) the presence of light chain and using goat anti-mouse IgM (Fig. 2c) the presence of heavy chain in transgenic callus tissue was demonstrated. The specific binding of Ac 38 (Fig. 2d) to the plant proteins adsorbed to the membrane indicates assembly of both chains to structurally intact antibodies providing the correct tertiary structure of the idiotope formed by both chains.

Western blotting using crude protein extracts was successful only in the case of callus tissue. Detection by the polyclonal anti-λ antibody revealed synthesis and processing of the chimeric precursor light chain (Fig. 3a). The observed band migrated at the same position as purified B 1-8 light chain (which is the processed form). No additional band providing a higher molecular mass which could represent a precursor light chain could be detected. This suggests that processing of the foreign light-chain precursor protein in plant cells is very efficient.

Analysis of leaf tissue was performed after wounding and incubation for 5 days with 10^{-5} M 2,4-D and 10^{-5} M Kinetin. This was done in order to obtain highest possible induced expression rates for both foreign genes (K. Düring, unpublished results). Purification by affinity chromatography with the NP hapten applied to protein extracts from induced greenhouse plant leaves confirmed the presence of assembled B 1-8. Binding of the antibody produced in plant cells to its antigen also implies its biological activity. Plant material was extracted and total protein incubated with a NP-Sepharose affinity gel. The binding affinity of B 1-8 to NiP (a related hapten; heteroclitic properties of B 1-8) is higher than to NP (the original immunizing hapten) [32]. Elution of the affinity gel with NiP released bound antibodies which were then loaded onto a denaturing western gel. The B 1-8 light chain could be detected by the polyclonal anti-λ antibody. This indirect analysis proves the functionality of the assembled antibody in plant extracts because isolated light chains do not interact with NP.

Extraction of plant tissue with a pure PBS solution produced a cross-reacting band on the

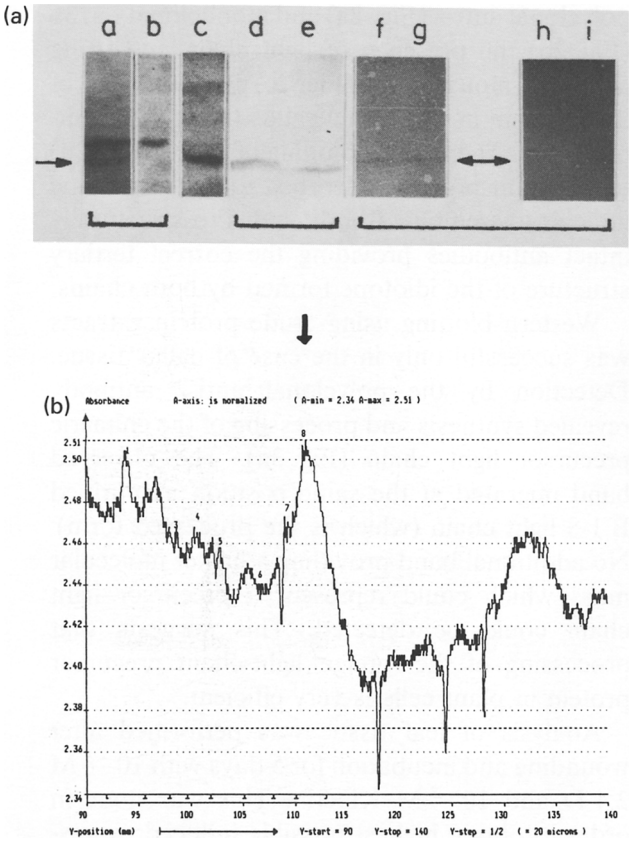

Fig. 3. a (top). Western blotting of transgenic tobacco tissue as described in 'Materials and methods', detailed description of experiments in 'Results'. *Lane a:* crude extract from callus regenerated from transformed leaf; *lane b:* crude extract from wild-type W38 callus; *lane c:* positive control: purified B 1-8 protein, processed light chain; *lane a–c:* all samples were extracted in denaturing SDS-sample buffer at 95 °C; detection of light chain with polyclonal anti-λ antibody; only processed light chain can be detected in transgenic tissue; *lane d:* demonstration of functionality of B 1-8 produced in induced transgenic greenhouse tobacco leaves by affinity chromatographic purification of the antibody with NP-Sepharose from plant PBS extract and subsequent denaturing western blotting; detection of light chain with polyclonal anti-λ antibody; upper band shows processed light chain; lower band: non-specific cross reaction; *lane e* as lane d: negative control with wild-type W38 leaves; *lanes f and g:* analysis of 10 mM CHAPS-PBS extract from induced greenhouse leaves by affinity chromatographic purification of the antibody and subsequent denaturing western blotting; detection of light chain with polyclonal anti-λ antibody; *lane f:* isolation of light chain with Ls 136-Sepharose; *lane g:* demonstration of functionality of B 1-8 produced in transgenic plant tissue with NP-Sepharose and detection of light chain; *lane h and i* as lanes f and g, respectively: negative controls with wild-type W38 leaves.

blot slightly lower than light chain which is also present in the wild-type tissue control (Fig. 3a, lane d + e). Addition of the detergent CHAPS to the extraction buffer prevented binding of this protein to the affinity gel but decreased band intensity on the blot (Fig. 3a, lane g + i). Additionally, the detection of light chain was confirmed by affinity chromatography from a CHAPS-PBS extract using the Ls 136 monoclonal antibody (Fig. 3a, lane f + h). Analysis of the blotting membrane (Fig. 3, lane g) by densitometer scanning corroborated the results obtained by eye visualization (Fig. 3b). Also in these experiments no precursor light chain could be detected. All bands detected were located at the same position as purified light chain.

Localization by electron microscopic immunogold labeling

Callus and stem tissue from transgenic tobacco plants induced by wounding and hormone application were analyzed by immunogold labeling at electron microscopic level following low-temperature preparation [23]. Ultrathin sections from callus tissue were incubated with biotinylated Ls 136 antibody and streptavidin-gold. Labeling was detected as striking accumulations of 3 to 10 gold particles in the cytoplasm (Fig. 4c) which were not found in the controls of wild-type tissue. Accumulation of several gold particles is typical of the biotin/streptavidin enhancement technique. Sections from callus and induced stem tissue were labeled with the monoclonal Ac 38 antibody and detected by colloidal gold-adsorbed anti-mouse IgG antibody. Assembled B 1-8 antibody could be detected in the endoplasmic reticulum (Fig. 4a) and surprisingly also inside chloroplasts often localized at thylakoid membranes (Fig. 4b; mostly 10–20 gold particles per chloroplast).

b (bottom). Densitometer scanning of the western blotting membrane, Fig. 3a lane g. Analysis was done on a LKB UltroScan XL laser densitometer (LKB, Bromma, Sweden); measurement without smoothing; the peak corresponding to the band at the size of mature processed light chain is indicated by an arrow.

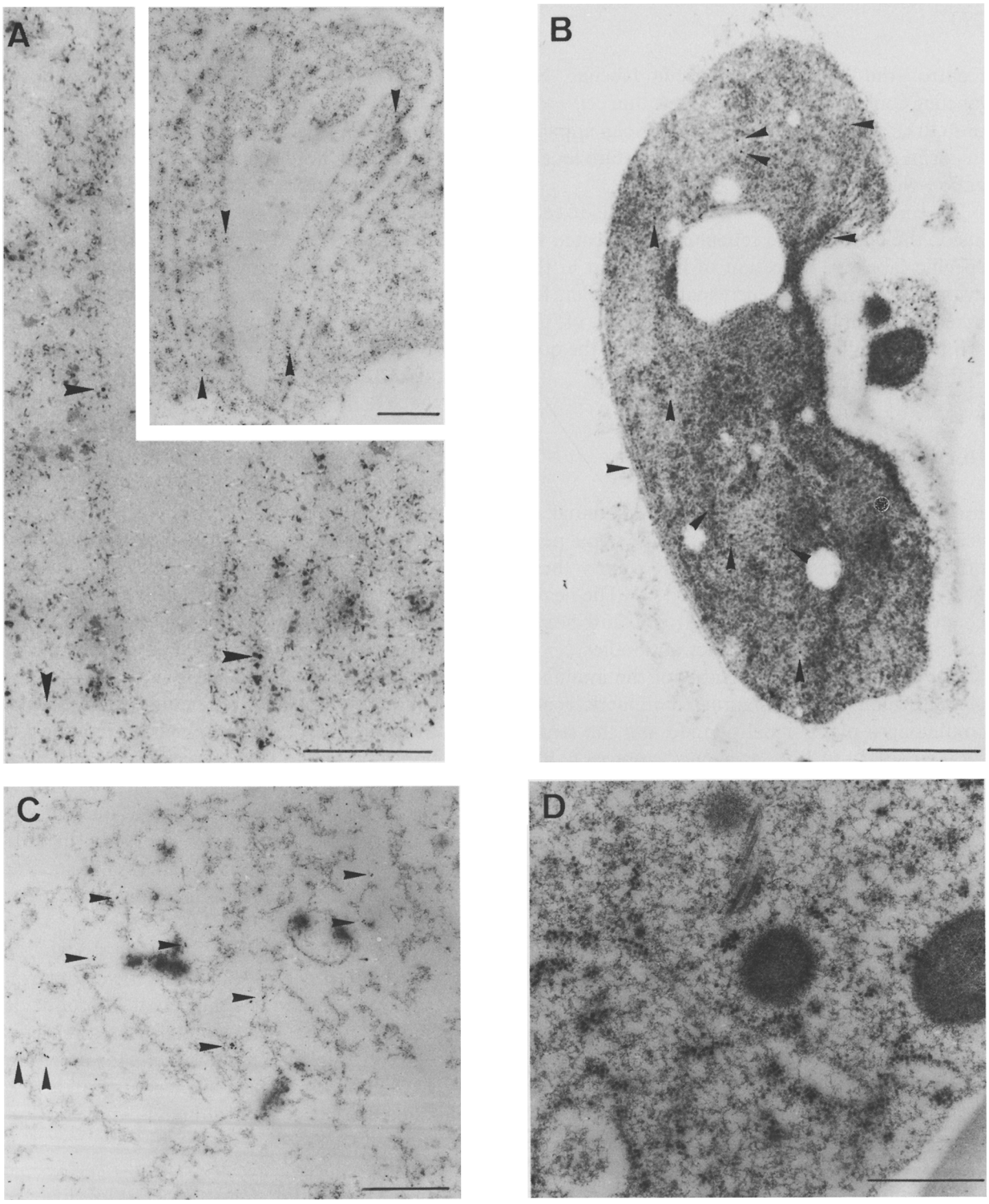

Fig. 4. Immunocytochemical localization of light chain and assembled antibody in sections of callus and induced stem tissue from transgenic tobacco plants. For details of electron microscopy see 'Materials and methods'.
A–C: transformed tissue; *A:* callus: labeled endoplasmic reticulum, detection of assembled antibody with monoclonal Ac 38 antibody; inset: enlarged overview on the endoplasmic reticulum section at lower magnification; *B:* lowest stem, induced: labeled chloroplast, detection of assembled antibody with monoclonal Ac 38 antibody; *C:* callus: cytoplasmic labeling, detection of light chain with monoclonal Ls 136 antibody; *D:* wild-type stem tissue: cytoplasm with endoplasmic reticulum, labeled with Ac 38; no unspecific labeling detected: bars = 0.5 μm.

Controls did not show any specific labeling. No labeling could be detected near or within cell walls or in the intercellular spaces, in the Golgi apparatus or in secretory vesicles. Fine structure localization will be continued.

Assembly of the B 1-8 antibody in plant cells inside the endoplasmic reticulum is indicated by specific labeling. Detection of light chain in the cytoplasm without any corresponding labeling for assembled antibody suggests that part of the synthesized light chain is separated from the normal secretory pathway.

Discussion

Immunoglobulins are synthesized in mammalian cells as precursors, containing specific signal peptides. After transport to the ER lumen these N-terminal extensions are cleaved off. The heavy chain is then glycosylated and light and heavy chains assemble to form intact antibodies.

For expression and assembly of the immunoglobulin subunits we constructed chimeric genes containing a plant signal peptide and the structural genes [13, 14]. A similar approach has already been taken for secretion of a chimeric T4 lysozyme in transgenic plants [15, 23]. The barley α-amylase signal peptide effected transport to the intercellular space. Using the same signal peptide we expected to find the immunoglobulin chains within the ER lumen. Comparable experiments in *E. coli* and yeast have shown that assembly of an active antibody is possible in both organisms only in the presence of signal peptides in the foreign precursors [1, 24, 36, 43]. Our data provide evidence that light and heavy chain are transported to the ER and that the subunits assemble within its lumen.

Detection of the synthesis and assembly of the monoclonal B 1-8 antibody in transgenic tobacco cells was performed by immunological methods. Tissue printing and western blotting provided data showing synthesis of both immunoglobulin chains as well as processing of the chimeric precursor light chain. The heavy chain could not be detected on western blots because of the lack of a specific and highly sensitive anti-antibody. Assembly of the antibody could be shown by tissue printing using an anti-idiotypic antibody. Binding of B 1-8 produced by plant cells to its Sepharose-bound hapten (NP) indicates functional activity. The bound antibody can be released by a related hapten (NiP) because B 1-8 shows heteroclitic properties. Detection of the light chain in this eluate demonstrates the presence of a functional antibody within plant cells. Individual light chains do not interact with the NP hapten [32]. Assembly of light and heavy chains in the ER is indicated by immunogold labeling using the Ac 38 anti-idiotypic antibody. Glycosylation of the heavy chain has not been investigated because of insufficient sensitivity.

Assembly of a stable active antibody involves formation of intramolecular disulfide bonds. This should be greatly facilitated in the endoplasmic reticulum because of the presence of disulfide isomerase. As described for a eucaryotic protein expressed in a procaryotic cell, the bacterial periplasmic space corresponding to the eucaryotic endoplasmic reticulum in the secretory pathway provides an environment facilitating formation of disulfide bridges since both compartments are involved in the first step of protein export. Spatial concentration within the ER may promote assembly to an active antibody. In addition, the important role of chaperonins such as BiP in protein folding and assembly is currently to be elucidated [6, 12, 19, 20, 26, 34]. Most probably similar plant chaperonins can be expected also in the ER but not in the cytoplasm. All these factors may be important in producing multimeric proteins as antibodies in transgenic plants. Nevertheless, in mouse cells the assembly of B 1-8 antibody lacking any signal peptide was obtained cytoplasmically although with very low efficiency [3].

Immunogold labeling at electron microscopic level provided additional data for the localization in the transgenic tissue. Binding of monoclonal Ls 36 antibody in the cytoplasm has to be due to the presence of individual light chains since no corresponding labeling could be found for an assembled antibody. This labeling may represent

a part of the light chain aborted from the secretory pathway. Garcia *et al.* [16] described an analogous phenomenon for the precore protein of hepatitis B virus. About 70–80% of the protein were found in an *in vitro* experiment in the cytosolic fraction although the signal peptide had been cleaved off implying a release of the processed protein from the endoplasmic reticulum. Immunoglobulin light chain may be produced in excess in relation to the heavy chain in transformed tissue because of higher promoter activity of the dual promoter directing the chimeric light-chain gene. Therefore part of the light-chain molecules always may remain unbound.

Localization of assembled antibodies in chloroplasts was detected unexpectedly. Significant amounts of gold particles were found with the monoclonal Ac 38 antibody inside these organelles. Control experiments with wild-type tissue did not show unspecific labeling. No convincing explanation can be given at the actual knowledge. No features of these engineered proteins were expected to direct an import of assembled antibody or individual immunoglobulin chains into chloroplasts. Therefore we cannot decide whether an import occurred or if other reasons cause this specific labeling. Immunogold labeling of transgenic tobacco tissue secreting a chimeric T4 lysozyme fused to the same α-amylase signal peptide revealed an unexpected localization of lysozyme in proplastids [15]. Elucidation of possible correlations requires further analysis.

No other labelings characterizing the secretory pathway or showing a final localization of the assembled antibody or its individual chains were found yet. Further fine structural analysis at electron microscopic level is under work.

In conclusion, the experiments described here provide evidence for the synthesis, insertion into the endoplasmic reticulum and assembly of immunoglobulin light and heavy chains as well as for the biological activity of the produced monoclonal antibody. Fusion of the immunoglobulin chains to a plant signal peptide may be the essential factor for production in transgenic plants when reviewing the actual state of knowledge concerning biosynthesis of antibodies in lymphoid cells. Expression of monoclonal antibodies in plants might be of importance in basic as well as in applied plant research.

In a paper just published by Hiatt *et al.* [22] a very similar approach for expression and assembly of a monoclonal antibody in transgenic plant tissue was described. In this case the natural mammalian signal peptides of light and heavy chains were used instead of a plant originating signal peptide. Analogous constructions without any signal peptide were also tested but only very low levels of single chains and no assembly could be detected in transgenic plant tissue. It was reported that expression of both precursor coding cDNAs in one plant resulted in formation of high levels of assembled antibodies. Data were obtained by ELISA and non-denaturing western blotting but not by affinity chromatography. The two chimeric genes were first introduced individually into different tobacco plants and then combined by sexual crossing. Parental plants expressing only one chain showed low levels of foreign protein. F1 plants expressing both chains revealed high levels of assembled antibodies. As RNA levels in all these plants were not significantly different light- and heavy-chain proteins have to be stabilized by aggregation.

Both strategies to assemble active monoclonal antibodies in transgenic plants were successful. Therefore we can conclude that the most important factor is the presence of a signal peptide fused to the mature light- and heavy-chain proteins and not its origin. In addition to the work described by Hiatt *et al.* we could demonstrate purification of the functional assembled antibody by affinity chromatography and localization by electron microscopic immunogold labeling. It was demonstrated that assembly indeed occurs in the endoplasmic reticulum also in transgenic plants. Both approaches may be useful also for assembly of other multimeric foreign proteins in plant tissue.

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