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**A novel chimeric MOMP antigen expressed in *Escherichia coli*, *Arabidopsis thaliana*,
and *Daucus carota* as a potential *Chlamydia trachomatis* vaccine candidate**

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Abstract

The major outer membrane protein (MOMP) of *Chlamydia trachomatis* is a highly antigenic and hydrophobic transmembrane protein. Our attempts to express the full-length protein in a soluble form in *Escherichia coli* and in transgenic plants failed. A chimeric gene construct of *Chlamydia trachomatis* serovar E MOMP was designed in order to increase solubility of the MOMP protein but with retained antigenicity. The designed construct was successfully expressed in *E. coli*, in *Arabidopsis thaliana*, and in *Daucus carota*. The chimeric MOMP expressed in and purified from *E. coli* was used as antigen for production of antibodies in rabbits. The anti-chimeric MOMP antibodies recognized the corresponding protein in both *E. coli* and in transgenic plants, as well as in inactivated *C. trachomatis* elementary bodies. Transgenic *Arabidopsis* and carrots were characterized for the number of MOMP chimeric genetic inserts and for protein expression. Stable integration of the transgene and the corresponding protein expression were demonstrated in *Arabidopsis* plants over at least six generations. Transgenic carrots showed a high level of expression of the chimeric MOMP—up to 3% of TSP.

1. Introduction

Chlamydia trachomatis (*Ct*) infection is a serious public-health problem. It is a cause of chronic conjunctivitis and is worldwide the most common sexually transmitted bacterial infection (STI) with more than 90 million new cases occurring annually [1]. Infection can result in scarring and fibrosis of ocular and genital tissues. The result is trachoma and pelvic inflammatory disease, respectively [2,3]. Chlamydial urogenital tract infections are treatable with antibiotics, but due to a high frequency of asymptomatic infections, control and elimination of the disease is difficult. There are indications that the risk of re-infection after antibiotic treatment of a previous infection is high – 13-26% [4]. Moreover, *Ct* enhances transmission of the human immunodeficiency virus (HIV) and may serve as a cofactor in human papilloma virus (HPV) infection [5,6]. This means that the control of *Ct* STIs may be possible only through the development of a safe and efficient vaccine. Such progress is slow but of high priority.

Major efforts in anti-chlamydial vaccine development are focused on subunit vaccines using the major outer membrane protein (MOMP) of *C. trachomatis* as the target antigen. MOMP is the most abundant and one of the most studied proteins for use as a *Ct* vaccine candidate [1,7]. It was shown that MOMP is able to induce both T-cell responses and neutralizing antibody production against chlamydial infection [8,9]. However, despite useful animal models, it has been difficult to achieve complete protection against *Ct* infection using anti-chlamydial subunit vaccines in animal experiments [1,9,10]. One probable reason for this is the use of an inefficient delivery system. Vaccine delivery is important in the case of STIs since mucosal immunity has to be achieved. Mucosal immunity can for instance be initiated through either the oral or the intranasal delivery route [11-14]. Plant-based edible vaccines or purified recombinant antigen protein for intra-nasal delivery are good candidates for mucosal

immunization. Especially plant-made proteins are generally safe and cheap, which opens up for a possibility to provide a high frequency of booster immunizations. Also, a transgenic plant is capable of producing several different antigens as a result of crossing parental lines producing different proteins.

The potential of the gut-associated lymphoid tissues (GALT) for induction of protective immune responses has hitherto only marginally been explored. Edible plant vaccines against enterotoxigenic *Escherichia coli* (ETEC; Refs. 14 & 15), cholera toxin [17,18], and norovirus [19, 20] have already passed pre-clinical trials and preliminary human clinical trials show very promising results – transgenic plants can stimulate a two-way immune response, both systemically and mucosally. Improvement of administration protocols and the use of adjuvants during oral vaccination could then be important ways of further increasing efficacy of edible vaccines.

The aim of this study was to develop a recombinant mucosal immunogen for *Ct* by combining two antigenic regions of the MOMP protein and decreasing the protein's hydrophobicity. The chimeric protein was overexpressed in *E. coli* and purified by immobilized metal affinity chromatography (IMAC). The genetic construct for this chimera was also introduced into the model plant *Arabidopsis thaliana* and into carrot (*Daucus carota*) and substantial production of the antigen was shown. The transgenic plants are planned for use as a production platform for the antigen or as edible vaccine vectors for laboratory animal experiments.

2. Material and methods

2.1. The MOMP constructions for overexpression in *Escherichia coli*

Total genomic DNA was isolated from a bacterial suspension (Örebro University Hospital, Örebro, Sweden), emanating from a *Chlamydia trachomatis* serovar E infected patient, using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

PCR amplification of the full-length MOMP for overexpression in *Escherichia coli* was performed using *Ex Taq* DNA polymerase (Takara Bio Inc, Shiga, Japan) and primers FL MOMP, forward 2 and FL MOMP, reverse 2 (Table 1). The PCR consisted of 35 cycles at 98°C (10 s), 55°C (30 s), and 72°C (2 min) followed by extension at 72°C (15 min). The PCR product was purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and cloned into the pET101 vector and verified by sequencing.

For the chimeric MOMP construct, the initial amplification of two DNA fragments (VS2 and VS4) of *Chlamydia trachomatis* MOMP, both containing B and T cell epitopes, was performed from the prepared genomic DNA using primers VS2,forward1, VS2,reverse1 and VS4,forward1, VS4,reverse1 (Table1). The PCR reactions utilized *Ex Taq* DNA polymerase (Takara Bio Inc, Shiga, Japan) and consisted of 35 cycles at 98°C (10 s), 55°C (30 s), and 72°C (1 min) followed by extension at 72°C (15 min). The PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and subjected to a second PCR performed under the same conditions as the first PCR but with primers VS2,forward2&3 and VS2,reverse2 for the VS2 extended fragment and VS4,forward2 and VS4,reverse2&3 for the VS4 extended fragment (Table1). The PCR primers for amplifying the VS2 and VS4 fragments also contained sequences for an amino acid linker [(Gly₄Ser)₂Gly₄] between the two domains. The purified extended VS2 and VS4 fragments were spliced by overlap extension [21] using the following conditions: 10 cycles at 95°C (1 min), 55°C (1 min), 72°C (2 min), followed by extension at 72°C for 15 min. The spliced

product was used for a third PCR utilizing Pfx Taq-polymerase (Invitrogen, Carlsbad, CA) and 25 cycles at 94°C (15 s), 55°C (30 s), 72°C (2 min) followed by a single extension step at 72°C (30 min). The last PCR amplification was performed using primers VS2,forward2&3 and VS4,reverse2&3 (Table. 1). The PCR product obtained was purified as described above.

2.2. Cloning and expression of the full-length MOMP and the MOMP chimera in *Escherichia coli*

The purified full-length MOMP DNA and chimeric MOMP construct were cloned into the pET101/D-TOPO[®] vector using the Champion pET Directional TOPO[®] Expression Kit (Invitrogen, Groningen, The Netherlands) according to the manufacturer's protocol (Fig. 1a & b). That our constructs were in frame with the C-terminal V5 and 6xHis fusion tags was confirmed by sequencing (ABI PRISM 310 GeneticAnalyser, Applied Biosystems, Foster City, CA). Each protein was expressed in the BL21 Star[™] (DE3) *E. coli* strain. A volume of 1000 ml of LB medium containing 50 µg/ml carbenicillin (Sigma-Aldrich, St. Louis, MO) was inoculated with 10 ml of a fresh overnight culture derived from a single colony of transformed *E. coli* and grown at 37°C to an optical density (OD) of 0.7 at 600 nm. Isopropyl β-D-thiogalactoside (IPTG; Invitrogen) was added to a final concentration of 1.5 mM, and the culture was further incubated for 4 hours. Bacteria were harvested by centrifugation (5000 x g, 15 min) and subjected to protein purification (see below).

2.3. Protein purification

The frozen bacterial pellet was first subjected to disintegration using an X-PRESS (AB BIOX, Göteborg, Sweden) with subsequent resuspension in 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich). After sonication on ice (35 W, 6 x 30 s) and ultracentrifugation (45000 x g, 45 min), two fractions were obtained: one soluble fraction and one insoluble fraction.

The soluble fraction was subjected to purification under native conditions using HIS-Select Nickel Affinity Gel (Sigma-Aldrich) according to the manufacturer's protocol. As equilibration and wash buffer, we used 50 mM sodium phosphate (pH 8.0) with 0.3 M NaCl. Elution was performed with the same buffer supplemented with a gradient of imidazole, the concentration of which ranged from 50 to 250 mM in 50 mM steps.

The pellet from ultracentrifugation containing the insoluble fraction was resuspended in 0.1 M sodium phosphate (pH 8.0), 8M urea and sonicated as described above. Insoluble material was removed by ultracentrifugation (50000 x g, 60 min). The supernatant was subjected to purification by IMAC under denaturing conditions according to the manufacturer's recommendations. The affinity gel was equilibrated with 0.1 M sodium phosphate buffer (pH 8.0) containing 8 M urea. The wash buffer was of the same content but had a pH of 6.3. Elution of the denatured proteins was again performed with the same buffer but with a pH of 4.5.

The collected fractions of the eluted protein were analyzed and the ones containing the protein of highest purity were pooled (separately for the native protein and for the denatured protein). The pooled fractions were concentrated by using an Amicon Ultra centrifugal filter device with a molecular weight cut off of 10 KDa (Millipore, Billerica, MA).

2.4. Production of anti-MOMP chimera antibodies in rabbits

Anti-MOMP chimera serum was produced in rabbit against the recombinant MOMP chimeric protein purified under native conditions (Davids Biotechnologie GmbH, Regensburg, Germany). The scheme of immunization of rabbits included six injections. On Day 0, 60 µg antigen was administered intradermally. On days 14, 21, 35, 49, and 63, 30 µg was given subcutaneously. Water-in-oil-emulsion (TiterMax; CytRx Corp, Los Angeles, CA) was used as adjuvant.

2.5. MOMP DNA constructs for plant transformation

PCR amplification of the full-length MOMP for expression in plants was performed with primers FL MOMP plant, forward 1 and FL MOMP plant, reverse 1 (Table 1) using total genomic DNA isolated from a bacterial *Chlamydia trachomatis* serovar E suspension as the template. The PCR was performed using Pfx Taq-polymerase (Invitrogen) and consisted of 35 cycles at 94°C for 30 s, 55°C for 60 s, 72°C for 3 min followed by a single extension step at 72°C for 30 min. The purified PCR product was subjected to subcloning into a plant expression vector (see below).

The chimeric MOMP was re-amplified from the previously obtained construct using primers VS2,forward2&3 and VS4,reverse,STOP (which introduced a stop codon into the product, Table 1) and Pfx Taq-polymerase (Invitrogen) to produce a blunt-end PCR product. PCR was carried out using the following conditions: 35 cycles at 94°C for 30 s, 55°C for 60 s, 72°C for 2 min followed by a single extension step at 72°C for 30 min. The PCR product was purified as previously described and used for subcloning into a plant expression vector.

As plant expression vector we used pGreen0229 (Ref. 22; <http://www.pgreen.ac.uk>) kindly provided by Dr. P. Mullineaux and Dr. R. Hellens, John Innes Centre and the Biotechnology and Biological Sciences Research Council (Norwich Research Park, UK). The expression cassette contained a *CaMV35S* promoter and a *CaMV* polyA terminator sequences, separated by a multi-cloning site. The vector was linearized by using the *Sma*I endonuclease at the multi-cloning site and used for cloning of the MOMP constructs. The resulting plasmids pGreen0229/chimeric MOMP and pGreen0229/MOMP were sequenced to confirm correct orientation of the inserts (ABI PRISM 310 GeneticAnalyser, Applied Biosystems).

2.6. Transformation of Arabidopsis

The pGreen0229/chimeric MOMP and pGreen0229/MOMP constructs (Fig. 1c & d) were used to transform *Agrobacterium* strain EHA105 (kindly provided by E.E. Hood, Department of Biology, Utah State University), by electroporation. Positive clones were selected on LB medium supplemented with kanamycin (50 µg/ml) and tetracyclin (5 µg/ml). *Arabidopsis thaliana* ecotype Columbia-0 (Col-0; The European Arabidopsis Stock Centre, Loughborough, UK) was used as background for plant transformation. After sowing on a fertilized soil:perlite:vermiculite mixture (1:1:1), seeds were maintained for 5 days at 4°C (darkness) and then transferred to a growth chamber (22°C, 16 h light, 8 h darkness, 70% humidity). The fluence rate of white light was 100 µmol photons m⁻² s⁻¹ (PAR). Transgenic plants were produced by the simplified floral dip method of four-week-old *Arabidopsis* as described by Clough and Bent [23] and selected by germination on Murashige and Skoog (MS) medium containing 10 µg/ml glufosinate-ammonium (BASTA; Riedel-de Haën, Seelze,

Germany) and 400 µg/ml cephotaxime (Sigma-Aldrich). Resistant plants were transferred to potting mix for analysis, self-pollination and seed production. The seeds obtained from individual plants producing 100% BASTA-resistant progeny were used for further experiments.

2.7. Transformation of carrot

Seeds of *Daucus carota* (L.) ssp. sativus cvs. Karotan and Napoli F1 (Weibulls trädgård AB, Hammenhög, Sweden) were sterilized in 25% [v/v] chlorine for 45 min and another 2 h in 2.5% [v/v] chlorine, 70% ethanol for 1 min, and, finally, washed three times in water during 1 h. Sterile *D. carota* seeds were germinated on MS medium without growth regulators and callus cells were initiated from excised hypocotyls by cultivation on MS medium with 2,4-dichlorophenoxyacetic acid (1 mg/l). The callus cells were suspended in liquid medium of the same type and grown in darkness on a shaker (90 rpm) at 25 °C. For production of somatic embryos, the cells were transferred to a growth regulator-free MS medium. For transformation, carrot cells were taken 10–14 days after addition of fresh growth medium. The carrot cells were packed by centrifugation (at 100 g for 1 min). 4–5 ml packed cells were diluted in liquid MS medium to 20 ml and 600 µl of *A. tumefaciens* carrying the vector pGreen0229/chimeric MOMP in LB medium (optical density 1.5 at 600 nm) was added. The cells and bacteria were co-cultivated for 3 days in darkness at 25 °C using a shaker (90 rpm). For selection of transgenic carrot cells, they were repeatedly washed three times by centrifugation in liquid MS medium to remove bacteria and were subsequently imbedded and further cultivated in growth regulator-free medium supplemented with BASTA (0, 1, 5, or 10 µg/ml) and cephotaxime (500 µg/ml) in dim light (1 µmol photons m⁻² s⁻¹) at 25 °C. The

density of carrot cells was 0.1–0.9 ml packed cells/10 ml of medium. Growing aggregates, and in some cases plants, were transferred to growth regulator-free MS medium without BASTA. The in vitro plants were cultivated and acclimated in 1 l plastic cans (PhytoTechnology Laboratories, Terrace Lenexa, KS, USA) in a mist-house for approximately 2 weeks giving 18 h/6 h light/darkness in dim light and, subsequently, cultivated in pots using the equal light period but with a light intensity of $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$.

2.8. Immunoblotting

To prepare protein samples, Arabidopsis tissue was ground in an extraction buffer containing 50 mM Tris, 8 M urea, 1% Triton X-100 and 1 mM DTT (pH 7.5). Carrot taproot tissue (about 200 mg) was ground in liquid nitrogen with a mortar and pestle. The frozen powder was thawed on ice and vortexed with 200 μl of 50 mM Tris-HCl buffer (pH 7.5). Protein extracts were separated by SDS-PAGE and blotted onto nitrocellulose membrane Hybond-C (Amersham Biosciences, Buckinghamshire, England). The membrane was blocked using 3% BSA (Sigma-Aldrich) in TBS (0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4) for 1 h and incubated with either mouse monoclonal antibodies raised against full-length *Ct* MOMP (Acris Antibodies GmbH, Germany) or anti-chimeric MOMP serum produced in rabbit against our recombinant protein for 1 h. Chimeric MOMP/primary antibody complexes were then detected with alkaline phosphatase (AP)-conjugated anti-mouse or anti-rabbit antibodies (Promega, Madison, WI) and visualized with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Promega, Madison, WI).

2.9. Genomic DNA extraction and Southern blot analysis

Analysis of genomic plant DNA for the number of transgenic inserts was performed only for *Arabidopsis* plants transformed with the chimeric MOMP construct. Plant genomic DNA was isolated using the JETFLEX Genomic DNA Purification Kit (GENOMED GmbH, Löhne, Germany), and 15 µg DNA was cleaved with either *Dra*I, *Nde*I or *Not*I (Sigma-Aldrich). These enzymes do not cleave the chimeric MOMP sequence. The cleaved DNA was separated by agarose (1%) gel electrophoresis and transferred to Hybond-N membrane (GE Healthcare, Uppsala, Sweden). The membranes were probed with chimeric MOMP DNA labelled with ³²P-dCTP using the random primers DNA labelling system (Invitrogen). The number of bands observed on the X-ray film corresponded to the number of T-DNA insertions in the plant genome.

2.10. Northern blot analysis

RNA isolation was performed according to Strid, Chow & Andersson [24]. Samples containing 15 µg of total RNA were electrophoretically separated on a 1.2% agarose gel and transferred to a Hybond-N membrane (GE Healthcare). The probe (full-length MOMP DNA) was labeled with ³²P-dCTP using the random primers DNA labelling system (Invitrogen). Blotting and hybridization was performed according to Kalbina and Strid [25].

2.11 Immunofluorescence analysis of antibody reactivity

To verify the reactivity of our anti-MOMP chimera antibodies produced in rabbits towards the full-length (intact) MOMP expressed by *Chlamydia trachomatis* bacteria, sera were analysed using an IgG/IgM Micro-Immunofluorescence Test kit against different *Chlamydia* species (ANI Labsystems, Vantaa, Finland) with minor modifications. Briefly, microscopic slides dotted with inactivated *C. trachomatis* elementary bodies were incubated with pre- and post-serum (1:64) from rabbits at 4°C overnight. Serum dilution buffer (PBS, 1% BSA) was used as a negative control for the conjugate. Glass slides were washed twice according to the manufacturer's recommendations and FITC-labeled goat polyclonal anti-rabbit-IgG antibodies (1:125; Abcam, Cambridge, UK) were incubated at 37°C for 30 minutes. The slides were analysed using a fluorescence microscope (Nikon Eclipse 80i, fitted with a Nikon PXM 1200F digital camera).

3. Results

3.1. Production of full-length MOMP in bacteria and in plants

Expression of the full-length MOMP in *E. coli* resulted in a protein that was present in insoluble form (not shown) and after lysis and ultracentrifugation the protein could be retrieved in the pellet only. Transgenic plants transformed with a full-length MOMP construct showed the presence of the transgene (PCR positive plants; not shown) and the MOMP mRNA (positive northern blot results; Fig. 2). However, there were no detectable MOMP protein neither in soluble or insoluble form (extraction with buffer containing 8M urea) as judged by immunoblot analysis using mouse monoclonal antibodies raised against *Ct* MOMP (Acris Antibodies; not shown). Therefore, our results indicate that the full-length *C.*

trachomatis MOMP could not be appropriately expressed in *A. thaliana*. Instead, we decided to design a MOMP-derived protein that was more likely to be expressed in plants and in *E. coli*.

3.2. The choice of constructs for production of *Chlamydia trachomatis* chimeric MOMP in bacteria and in plants

Since production of full-length MOMP was not straight-forward, neither in *E. coli*, nor in plants, a fact that is most likely due to its high content of hydrophobic amino acids, primarily reflected by the presence of 16 transmembrane helices, we wanted to produce a smaller and more hydrophilic protein based on MOMP but which still would retain high antigenicity. Therefore, we used the putative secondary structure described by Findlay *et al* [26] for this design and selected large parts of the VS2 and VS4 domains of the MOMP structure (Fig. 3a). These domains contain clusters of previously described T and B cell epitopes important for a protective immune response against *Ct* [27-31]. This includes also minor stretches of the transmembrane part of the protein, in the vicinity of the loops, since these hydrophobic stretches also contain immunogenic epitopes. In addition, the choice of domains was such that the difference between the primary structure based on *Ct* serovar E only differed marginally (6 amino acid residues out of 99) from that of serovar D (Fig. 3b), making it highly likely that the chimera would induce an immune response to both serovars if used as a candidate vaccine antigen. Finally, the choice of an amino acid linker (Fig. 3b) between the two domains and the retained hydrophobic amino acid residues was such that we could envisage two different tertiary structures of the MOMP chimera, one flexible structure (Fig. 3b) and a more rigid

structure (Fig. 3c), respectively, again maximizing the chimera's function as a vaccine antigen.

3.3. Chimeric MOMP construct and its expression in *E. coli*

The reverse and forward primers used in PCR to amplify the VS2 and VS4 variable regions of MOMP for assembling the chimera were designed from the nucleotide sequence data. The sequence encoding a common flexible linker, [(Gly₄Ser)₂Gly₄], was introduced into the 5'-end of the VS4,forward2 and VS2,reverse2 primers. The amplified VS2 and VS4 fragments were then assembled as follows: 5'-VS2 – linker – VS4-3' (Fig. 3b). The genetic construct produced showed the expected size of 351 bp (Fig. 4a). The product was verified by sequencing and cloned into the pET101 vector (containing sequences encoding C-terminal V5- and His-tags; Fig. 3b). The expressed protein was detected using both anti-His antibodies (data not shown) and anti-full length MOMP antibodies (Acris Antibodies; Fig. 4b). Typically, in a 6000 ml *E. coli* culture, 70-80 µg per ml of MOMP chimera was obtained with approximately 5% in soluble form, yielding a total of some 20 mg of soluble MOMP chimera protein.

For purification of the MOMP chimera using IMAC technology, we expressed the protein in 2000 ml bacterial cultures. The soluble chimeric protein was purified under both native and denaturing conditions. The elution fractions of chimeric MOMP protein, were purified under native conditions, analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue (not shown). Pure fractions were pooled and were later used in immunization experiments for production of anti-chimeric MOMP polyclonal serum and thereby for verification of immunogenic features of the designed MOMP chimera. Freshly prepared

MOMP chimera ran as a monomer on SDS-PAGE (Figs. 4 & 5) whereas the corresponding protein that had been stored in the refrigerator for several months and used as positive controls ran as a dimer (Fig. 8a). Proteins that had been stored for a few months displayed both bands on SDS-PAGE gels (Fig 8b).

3.4. Production of anti-MOMP chimera antibodies in rabbit and immunofluorescence analysis

The antibodies produced against the native chimeric MOMP were tested against the purified recombinant MOMP chimera. As shown in Fig. 5, the anti-serum recognized a band of the correct size. At the same time, the pre-serum did not recognize any bands. Affinity chromatography-purified anti-serum did not show a stronger signal to the goal protein (not shown) than the antiserum with lower antibody concentration.

Since the final aim of our project is to obtain an antigen suitable for vaccination, it is important to show that the antibodies raised using the MOMP chimera do recognize the native full-length *Ct* MOMP protein. Toward this end, immunofluorescence using our anti-MOMP chimera antibodies, produced in rabbits (post-serum), were used to study reactivity towards *Ct* elementary bodies. High reactivity was obtained as demonstrated by the clearly defined fluorescent dots in Fig. 6a. The rabbit pre-serum did not show specific reactivity towards these *Ct* elementary bodies (Fig. 6b). Furthermore, the conjugate itself did not contribute to unspecific binding (fluorescence). This was demonstrated in negative controls without incubation with rabbit serum (Fig. 6c).

3.5. MOMP chimera production in *Arabidopsis* and analysis of the transgene

The designed MOMP chimera was ligated into the *Sac*I cloning site of the pGreen vector, and the sequence of the cloned fragment was verified. The recombinant expression vector was used to transform *A. thaliana* plants of the Col-0 ecotype. Forty transgenic plants were selected after initial seedling screening with BASTA. Three selected transgenic lines (numbers 9, 15 and 25) were used in further analysis and stable integration of the transgene in these lines was demonstrated for up to six generations using the polyclonal antibody against *C. trachomatis* MOMP (Acris Antibodies; Fig. 7a). Whereas both transformed and wild type *Arabidopsis* showed a false positive band with a size of approximately 25 kDa, a specific band of the correct size that fits well with the size of the *E. coli*-expressed recombinant protein was found in transformed plants only.

The transgenic plants chosen were subjected to Southern blot analysis in order to estimate the number of transgenes. Restriction enzymes *Dra* I, *Nde* I, and *Mlu* I were used for cleavage of plant genomic DNA. The results obtained with *Dra* I and *Nde* I are shown in Fig. 7b. Different numbers of transgene insertions occurred in the different lines: line 9 contained one insert, line 12 three, line 15 two, and line 25 four inserts. Although different numbers of the transgene was present in different lines, this did not visually influence the phenotype of the plants. The transformants had an identical appearance compared with the *A. thaliana* wild type (WT) plants.

3.6. MOMP chimera production in carrot

MOMP chimera production using *Daucus carota* was also analysed by immunoblotting with monoclonal antibodies to Ct MOMP (Acris Antibodies). Fig. 8a shows the results of a semi-quantification of the amounts of MOMP chimeric protein produced using cultivar Karotan (line Kar +; denoted Kar in Fig. 8a) and cultivar Napoli (line 313/3; denoted 313 in the same Fig.), and compared with standard amounts of our *E. coli*-produced MOMP chimeric protein (180, 300, 600, and 1200 ng). The line Kar + produced approximately 450 ng MOMP per 40 µg total soluble protein (TSP), corresponding to 1%. The line Napoli 313/3 produced approximately 600 ng MOMP per 20 µg TSP, corresponding to 3%. As was the case with *E. coli*-produced chimeric MOMP that had been stored in the refrigerator for several months, the protein expressed in carrots always ran as a dimer on SDS-PAGE (Fig. 8).

The antiserum raised against the *E. coli*-produced native chimeric MOMP was also tested with plants expressing the transgene. The antiserum recognized the dimeric form of the protein in transgenic carrot (Fig. 8b) but not in the wild-type, whereas the monomer was found in transgenic *Arabidopsis* lines (not shown). The antibodies are obviously specifically labelling the plant-produced chimeric MOMP.

4. Discussion

The objective of this study was to create an antigen candidate that could be used for immunization against infection by *Chlamydia trachomatis* serovars E and D, primarily in laboratory animals, and to express antigen *in planta* (*Arabidopsis thaliana* and carrot) as a

putative oral vaccine. Finally, we wanted to produce antibodies against the MOMP protein in rabbits to show the protein's potential antigenicity and to be able to use these antibodies as an analytical tool for future studies.

During the course of this study we did not succeed in expressing the full-length MOMP protein in *Arabidopsis thaliana* plants. Even though we had evidence for the presence of both the transgene (positive PCR) and its transcripts (positive northern blotting results) *in planta*, we were unable to detect the MOMP protein in plants. This is most likely due to its strong hydrophobicity. The MOMP protein topology was modelled as a 16-stranded membrane-bound β -barrel [26]. In the full-length protein, 128 out of 371 amino acids belonged to the transmembrane part of the protein (34.5%). Expression of MOMP in heterologous systems such as *E. coli* has also previously proved to be highly problematic, since the protein tends to misfold and aggregate [32], a result that was also repeated in our study.

Due to these severe problems with expression of the full-length MOMP, another approach was taken. The new design was based on an analysis of the entire MOMP sequence and thereby merging of certain highly antigenic regions of MOMP to form a chimeric polypeptide, and at the same time minimization of the number of hydrophobic amino acids belonging to transmembrane helices. We have combined in our construct both epitopes important for a cell-mediated immune response (T helper cells and cytotoxic T-lymphocytes) as well as neutralizing antibodies, which are necessary for the creation of a protective immune response against *Ct*. T-cell stimulating epitopes for human leukocyte antigen (HLA) class I and HLA class II recognition, that are mainly situated in the constant domains (CDs) of the MOMP [27], are included in the chimera. The chimera also contains epitopes for antibody recognition that are present in the variable domain regions (VDs) of MOMP (Fig. 3a; Ref. 28). However, some small hydrophobic stretches containing immunogenic epitopes were kept

in the new chimera (see Fig. 3). Also, we wanted to express a chimeric protein, based on the serovar E amino acid sequence, that was as similar as possible to the serovar D sequence, with the aim to produce an antigen candidate protein that would be able to evoke an immune response against both serovars. In this way, we could use the serovar D-based animal model of our research partners to study the potential of our construct to cause cross-serovar protection (work in progress).

Again, the chimera contains hydrophobic parts of three transmembrane helices partly since important peptides for T-cell activation are located there and since it is necessary to obtain a stimulatory T-cell response in order to obtain a functional vaccine against *Ct* [29], but also partly since clustering of these hydrophobic segments could potentially present the antigen in a form that resembles the original tertiary MOMP structure and thereby would be more likely to induce a useful immune response. Therefore, some hydrophobic amino acids were kept in the chimeric MOMP. We are aware that the inclusion of these short hydrophobic stretches into the primary structure of our MOMP chimera does not necessarily induce a stable or immunogenic conformation. However, our results do show that the chimera indeed fulfils its task, i.e. ease of production and purification and induction of synthesis of functional antibodies against the full-length MOMP: the rabbit antibodies we raised using the chimeric MOMP recognize full length MOMP in *Ct* elementary bodies (Fig. 6).

Notwithstanding, our designed chimera would be considerably more soluble than the full-length MOMP and therefore more readily expressed in transgenic plants. In fact, in the novel chimeric construct, the VS2 and VS4 loops and the linker comprised 75% of the polypeptide, the hydrophobic residues of transmembrane part of the full-length MOMP (according to the model described by Findlay et al [26]), only being 19% of the amino acid content. Indeed, successful expression of the MOMP chimera was obtained in all three systems (*E. coli*, *Arabidopsis*, and carrot).

In fact, stable integration of the transgene was demonstrated in *Arabidopsis* over at least six generations, which was proven by immunoblot analysis (Fig. 7a) and in carrot we were able to achieve a high expression level of chimeric MOMP – up to 3% of TSP. The stability of the transgene in the offspring is important for the possibility of scaling up transgenic plant production. As was demonstrated by Lindh et al. [33, 34], both *A. thaliana* and carrot are eaten raw by mice and therefore can function as model immunization vectors in immunological and challenge studies, as well as in pre-clinical trials. Animal experiments using transgenic *Arabidopsis* plants for oral administration are under way, as well as experiments using purified chimeric MOMP for intranasal mucosal administration.

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6. References

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Figure legends

Fig. 1. Schematic view of the constructs used in this study: a) and b) denote full-length MOMP and chimeric MOMP, respectively, expressed in *E. coli* (pET101/D-TOPO vector), c) and d) denote full-length MOMP and chimeric MOMP, respectively, expressed in plants (pGreen0229 vector).

Fig. 2. Northern blot analysis of plants transformed with the full-length MOMP construct. Plants 1 and 2 show the presence of MOMP mRNA transcripts. WT denotes untransformed wild type plant. All three tested transgenic plants were PCR positive.

Fig. 3. a) Topology and primary structure of the *Ct* serovar E MOMP as adopted from Findlay et al. [23]: squares, amino acids residues found in membrane spanning helices; circles, amino acids residues found in extramebraneous parts of the protein. The domains selected for design of the chimeric MOMP are shown in red; b) The putative flexible conformation that can be obtained using the (Gly₄Ser)₂Gly₄ linker (shown in black). The amino acid residues that differ between MOMP serovar E (shown) and serovar D in the VS2 and VS4 loops are given in blue; c) The more rigid conformation that can be obtained using the (Gly₄Ser)₂Gly₄ linker (shown in black). The amino acid residues that differ between MOMP serovar E (shown) and serovar D in the VS2 and VS4 loops are given in blue. The green C-terminal tag contain a V5 epitope and a His₆ purification tag, as expressed in *Escherichia coli* but not in plants (see Fig. 1).

Fig. 4. a) PCR analysis of the assembled MOMP chimeric construct. Ch denotes PCR product from a vector containing the assembled chimera, N denotes the PCR negative control, L

denotes the DNA size marker. The amplified product has the expected size of 351 bp.; b) Western blot analysis of recombinant His-tagged chimeric MOMP protein expressed in *Escherichia coli* and purified using Ni-NTA chromatography. A band of the expected size (17 kDa) was detected using mouse monoclonal antibodies to *Chlamydia trachomatis* MOMP (Acris Antibodies). Ch denotes the chimeric MOMP protein, L denotes the protein size marker.

Fig. 5. Evaluation of the anti-chimeric MOMP antiserum produced in rabbits. The purified recombinant MOMP chimera was analyzed by immunoblotting using anti-chimeric MOMP serum (S), affinity purified anti-chimeric MOMP antibodies (A) and pre-serum (P). L denotes the protein size marker.

Fig. 6. Immunofluorescence slides demonstrating antibody reactivity toward *Chlamydia trachomatis* elementary bodies and its full-length MOMP protein (bright fluorescent dots). a) Anti-MOMP chimera antibodies (post-serum), produced in rabbits injected with MOMP chimera, showing high specific reactivity against inactivated *Ct* elementary bodies. b) Rabbit pre-serum lacking MOMP reactivity. c) Minimal fluorescence of the secondary anti-rabbit IgG antibody conjugate itself in the absence of rabbit serum. Magnification was 400x in a) and 200x in b) and c), respectively.

Fig. 7. a) Western blot detection of constitutively expressed chimeric MOMP in *Arabidopsis* leaf extracts from T6 generation plants using polyclonal antibody against full-length *C. trachomatis* MOMP (Acris Antibodies). L denotes the protein size marker; 9, 15 and 25 denote three different transgenic lines of *Arabidopsis*; WT denotes non-transformed wild type *Arabidopsis*; A corresponds to 5 µl unfractionated plant extract and B corresponds to 15 µl

unfractionated plant extract; b) Southern blot analysis of four *Arabidopsis* lines transformed with the chimeric MOMP construct (lines 9, 12, 15, and 25). Two different DNA digests of each line were produced by using the *Dra* I and *Nde* I restriction enzymes and probed with random primer ³²P-labelled chimera MOMP oligonucleotides. The restriction enzymes chosen did not digest the MOMP chimera transgene itself. The number of observed bands corresponds to the copy number of the transgene.

Fig. 8. a) Semiquantitative analysis of the content of chimeric MOMP in transformed carrots. Kar and 313 denote two different transgenic lines in cultivars Karotan and Napoli, respectively. Comparison of the intensity of the stained bands in the transgenic plants and controls (purified and accurately quantified chimeric MOMP) allowed the estimation of the approximate MOMP chimera protein concentration in the carrots. b) Immunoblot showing the specificity of the antiserum raised against *E. coli*-produced chimeric *Ct* MOMP protein when used for probing extracts from carrot lines 350 and 640 (in the Karotan background) expressing the same protein. L denotes the molecular weight standards, WT are extract from wild type Karotan carrots, and PC are *E. coli*-produced positive controls (2.5 and 7.5 µg protein, respectively). The asterisks indicate the MOMP chimera dimer.

Table 1. Nucleotide sequences of primers used for PCR cloning of the full-length and chimeric MOMP antigens.

Primer name	Sequence (5' → 3')
FL MOMP, plant forward 1	TAGAACGGATCCTATGAAAAAACTCTTGAAATCGG
FL MOMP, plant reverse 1	CAAGATGGATCCGTTAAACTGTAACTGCGTATTTGTCTG
FL MOMP, forward 2	ATGAAAAAACTCTTGAAATCGG
FL MOMP, reversed 2	AACTGTAACTGCGTATTTGTCTG
VS2,forward1	TATTTGGGATCGCTTTGATGTAT
VS2,reverse1	TATTGGAAAGAAGCCCCTAAAGT
VS4,forward1	CTCTTGCACTCATAGCAGGAACT
VS4,reverse1	TGTAAGTGCCTATTTGTCTGCAT
VS2,forward2&3	CACCATGGGAGATAATGAAAA
VS4,reverse2&3	GGAGACGATTTGCATGGTAT
VS4,forward2	CAGGCGGAGGTGGATCCGGCGGTGGCGGATGGCAAGCAAGTTTAGCTCTCTCT
VS2,reverse2	CCGCCGGATCCACCTCCGCCTGAACCGCCTCCACCAAGTTCAACAACAGATTGATCT
VS4,reverse,STOP	ATTGAGCTCGCCTCAGGAGAC

1**2****3****WT**

Highlights

- We designed a *Chlamydia trachomatis* MOMP chimera containing major antigenic epitopes
- The chimera was successfully produced in *E. coli*, carrot and *Arabidopsis thaliana*
- *Arabidopsis* plants stably express the MOMP chimera over at least six generations
- Purified MOMP chimera retained antigenicity when injected into rabbits
- Anti-MOMP chimera antibodies was reactive against *C. trachomatis* elementary bodies