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Production of a recombinant bacterial lipoprotein in higher plant chloroplasts

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Little is known about the potential of plastids to accomplish post-translational modifications of foreign proteins. In the present study we generated transplastomic tobacco plants that accumulate the outer surface lipoprotein A (OspA)—the basic constituent of the first generation monovalent human vaccine against Lyme disease. The recombinant OspA exhibits a lipid modification typical for bacteria and induced protective antibodies in mice, demonstrating that functionally active bacterial lipoproteins can be processed in plants.

During the past 15 years transgenic plants have been shown to be suitable for large-scale and cost-saving production of therapeutics for application in humans and animals[1]. A number of model plants have been successfully transformed to produce complex structures with native conformation such as vaccine subunits[2]. Moreover, transgenic plants have already been shown to be suitable for oral immunization, including of humans[3]. But nuclear transformation of plants often results in low yield of the recombinant proteins, and so other protocols, in particular transformation of the chloroplast genome, have been pursued[4]. However, very little is known about the chloroplast's abilities to modify foreign proteins post-translationally.

In the present study we have expressed the gene encoding the bacterial lipoprotein OspA from *Borrelia burgdorferi* in tobacco chloroplasts. OspA is a prototype bacterial lipoprotein, having a distinctive lipid modification at its N terminus, which has not been observed in eukaryotic cells[5]. OspA, which has been widely used as a Lyme disease vaccine[6], needs to be lipidated to be immunogenic and to induce protective antibodies[7]. Here, we examined the lipid modification of OspA after chloroplast expression.

Two primary constructs were cloned for evaluating chloroplast expression of *ospA*: first, the full-length *ospA* gene, including the N-terminal signal sequence[8] and a C-terminal hexahistidine extension for subsequent purification and, second, a truncated version of *ospA* termed *ospA-T*, which lacks 45 base pairs at the 5′-end, resulting in the replacement of the first 16 N-terminal amino acids by a single methionine (see **Supplementary Methods** online).

From several independent transplastomic plants identified by Southern blot analysis (data not shown), one representative line of each construct, line *Nt*-pOspAhis-13 (accumulating recombinant chloroplast (rcOspA) and line *Nt*-pOspA-T-2 (rcOspA-T), were chosen for subsequent analysis. Leaf tissue was taken from greenhouse-grown plants and the amount of rcOspA/rcOspA-T present in crude plant extracts was estimated by immunoblot analysis of serial dilutions and compared to a purified preparation of *Escherichia coli*-derived recombinant OspA (rbOspA)[9] (see **Supplementary Fig. 1** online). The amount of recombinant proteins in tobacco protein extracts was calculated for rcOspA to be ~1% and for rcOspA-T ~10% of total soluble protein.

To determine whether rcOspA or rcOspA-T are lipidated *in planta*, we incubated isolated protoplasts of transgenic plant lines in the presence of ^{3}H-palmitic acid. Cell lysates were immunoprecipitated with anti-OspA antibodies and precipitates were subjected to gel electrophoresis and analyzed by fluorography. ^{3}H-palmitic acid was only incorporated into rcOspA, but not rcOspA-T (**Fig. 1a**). Since rcOspA-T was not palmitoylated, in spite of a cysteine residue in position 2 of the polypeptide chain, spontaneous attachment of palmitic acid via a thioester bond seems to be unlikely.

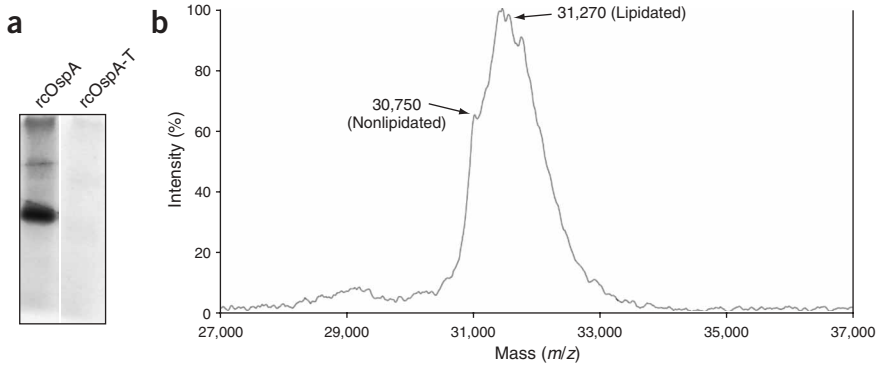

Figure 1 Analysis of rcOspA. (**a**) Fluorography of recombinant proteins rcOspA and rcOspA-T after metabolic labeling of isolated protoplasts with ^{3}H-palmitic acid and immunoprecipitation. (**b**) MALDI-TOF mass spectrum of rcOspA. The spectrum of the singly charged monomer revealed the heterogeneity of the component, in agreement with the expected mass and assuming a dipalmitoylated moiety attached to the protein (theoretical mass for a full-length lipidated protein, 31,272).

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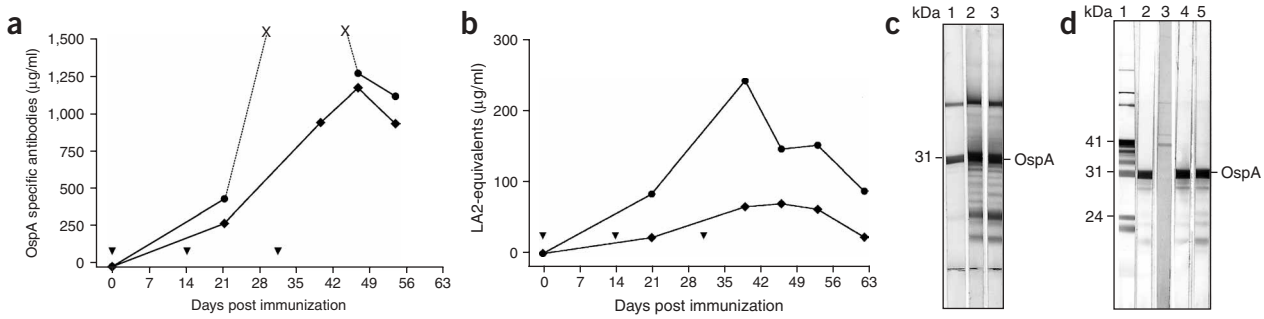

Figure 2 Immunogenic properties of rcOspA. BALB/c mice were immunized with chloroplast-derived rcOspA (♦) or *E. coli*-derived rbOspA (●) on days indicated (▼) (animal experiments were licensed by Reg Präsid. Freiburg, Si-5; Reg Nr. G-05/03). (**a**,**b**) Serum pools ($n = 3$/group) were taken at different time points and OspA-specific antibodies (**a**) or LA2-equivalents (**b**) were determined by enzyme-linked immunosorbent assay. x, value out of range. (**c**) Western blot analysis of serum pools taken on day 21 post immunization on recombinant OspA, 250 ng/lane. Lane 1, BALB/c anti-OspA IS (reference serum); lane 2, BALB/c anti-rbOspA IS; lane 3, BALB/c anti-rcOspA IS. (**d**) Western blot analysis of serum pools taken on day 21 post immunization on *B.b.* ZS7-Lysate, equivalent 2×10^6 *B.b.*/lane. Lane 1, standard monoclonal antibody mix; lane 2, BALB/c anti-OspA IS (reference serum); lane 3, BALB/c normal serum; lane 4, BALB/c anti-rbOspA IS; lane 5, BALB/c anti-rcOspA IS. *B.b, Borrelia burgdorferi.*

Purified rcOspA (see **Supplementary Methods** online) was further analyzed by matrix-assisted laser desorption ionization/time-of-flight/mass spectrometry (MALDI-TOF-MS). The mass spectrum of the singly charged ion (**Fig. 1b**) indicated the presence of a heterogeneous component, the main fraction of the molecule being lipidated (*m/z*, 31270). The measured mass for this component (*m/z*, 31270) was in agreement with the calculated mass for a full-length protein (including the N-terminal signal sequence) modified with a dipalmitoyl glycerol moiety. The broad peak could be due to variations in fatty acid saturation and chain length, which was also observed with the bacterial lipoprotein[5]. Furthermore, oxidation of the diacylglycerol moieties, and of the protein itself might be responsible for a heterogeneity in masses[5], a possible explanation for difficulties in resolving each component.

Hence, rcOspA derived from plants still possesses the N-terminal signal sequence. In the case of gram-negative bacteria, the N-terminal signal sequence channels the pro-lipoprotein to a biosynthetic pathway, which ultimately yields the unique lipid-modified amino acid, N-acyl-diacylglycerylcystein (Pam3Cys)[10]; similar pathways exist in gram-positive bacteria and in cyanobacteria[8,11]. Plant chloroplasts seem to have retained the ability to accomplish at least the first step of this unique pathway. This is possibly a heritage of their bacterial ancestors, although analysis of the public available genome sequences did not reveal any orthologs of the bacterial gene encoding for the modifying enzyme.

For analysis of the immunogenicity of rcOspA, in particular its potential to induce antibodies, the enriched preparation was lyophilized and subsequently resolved in phosphate buffered saline (PBS). BALB/c mice were repeatedly injected subcutaneously in the presence of adjuvant with a given amount of rcOspA or, as a control, with lipidated *E. coli*-derived rbOspA[12], the formula of the first generation vaccine against Lyme disease[9,13]. Both preparations, rcOspA and rbOspA, induced similar amounts of serum OspA-reactive antibody over a period of 56 d post injection (p.i.) (**Fig. 2a**,**b**), with one exception (day 40 p.i.; rcOspA, 850 µg/ml; rbOspA, 2,900 µg/ml). The specificity of antibodies generated by the indicated protocol was confirmed by western blot analysis of the respective immune sera (IS, taken at day 21 p.i.), using both rbOspA (**Fig. 2c**) and *B. burgdorferi* cell lysate (**Fig. 2d**). When compared for the level of serum antibodies directed to the dominant protective LA-2 epitope of OspA[6,12] (**Fig. 2b**), both IS were found to contain the respective specificity, however, at somewhat lower levels (two- to threefold) in IS rcOspA (~one-tenth of all OspA-specific antibodies; up to 60–70 µg/ml at days 39–54 p.i.) as compared to that of rbOspA (~one-fifth of all OspA-specific antibodies).

Previous findings showed that the induction of 0.5 µg/ml of LA-2-related OspA-reactive antibodies with *E. coli*-derived rbOspA was sufficient to protect mice against a subsequent challenge with *B. burgdorferi*[14]. In this study, the chloroplast-derived rcOspA generated protective antibodies significantly above that threshold, therefore suggesting that lipid-modified recombinant OspA from chloroplasts may also be used to provide protection against Lyme disease. This demonstration that functionally active bacterial lipoproteins can be processed in chloroplasts, suggests the presence of a genetic and enzymatic basis for lipidation in plants.

Sequences reported herein are deposited in GenBank with accession numbers DQ160002 and DQ160003.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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