

Enhanced expression of the human CD14 protein in tobacco using a 22-kDa *alpha*-zein signal peptide

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Abstract The human CD14, a high affinity receptor for lipopolysaccharides (LPS), is involved in the innate immunity system and the inflammatory response. There is increasing interest in using recombinant approaches to produce purified CD14 protein for therapeutic uses. Plants provide ideal expression systems for the production of recombinant proteins, but the levels of expression of recombinant proteins produced in *planta* are still not high. To improve expression levels of CD14 the 22-kDa *alpha*-zein signal peptide (ZSP) from maize was fused to the human *CD14* cDNA so that recombinant CD14 could stably accumulate in plant cells. The human *CD14* gene and the modified human CD14 cDNA with the 22-kDa ZSP were respectively transformed into tobacco to produce transgenic plants. Western blot analysis confirmed human CD14 accumulation in the transgenic tobacco. The concentration of the recombinant protein in the tobacco leaves was measured by ELISA, and the results suggested that fusion with the 22-kDa *alpha*-ZSP effectively increased the accumulation of the recombinant protein (rCD14). The concentration of rCD14 in some of the transgenic lines was 19.54 $\mu\text{g g}^{-1}$ tobacco leaf (fw), which was about 0.6 % of the total soluble protein. The rCD14 protein showed natural LPS-binding bioactivity by using U937 cells mensuration. Our results suggested that the maize 22-kDa *alpha*-zein

signal peptide could be used to increase the accumulation of recombinant protein in tobacco leaves so that proteins can be produced in abundant biomass.

Keywords CD14 · *Alpha*-zein signal peptide · Transgenic plant · Expression level · Molecular farming

Abbreviations

<i>als</i>	Acetolactate synthase gene
CaMV	Cauliflower mosaic virus
<i>eps</i>	5-Enolpyruvyl-shikimate-3-phosphate synthase gene
fw	Fresh weight
GPI	Glycosylphosphatidylinositol
LPS	Lipopolysaccharide
mCD14	Membrane-associated CD14
MW	Molecular weight
PSV	Protein storage vacuole
rCD14	Recombinant CD14
sCD14	Soluble CD14
WT	Wild type
ZSP	Zein signal peptide

Introduction

CD14 was first described at the First Leukocyte Typing Workshop in 1982 (Bernard et al. 1984). It exists in two main forms: one is a 53–55 kDa membrane-associated form (mCD14), and another is a soluble form with 48–50 kDa molecular weight (Bazil and Strominger 1991). CD14 functions as a high-affinity receptor for the lipopolysaccharides (LPS) of Gram-negative bacteria or the peptidoglycan of Gram-positive bacteria (Wright et al.

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1990). With the assistance of LPS-binding protein (LBP), CD14 can bind to LPS and trigger an inflammatory response (Wright et al. 1990; Blais et al. 2005).

During the past 20 years, the ability of sCD14 to protect against infection and its therapeutic potential have been described in numerous studies both in vitro and in vivo. Recently some preventive applications of sCD14 have been used, such as adding the protein to contact lens solutions, or to commercial milk formulas, to prevent the mucosal penetration of Gram-negative bacteria and improve innate immunity (Blais et al. 2006). CD14 has attracted more and more interest from the public. Therefore, we focused on producing low cost CD14 with genetic-engineering technology.

Plants are now considered as viable and competitive expression systems for the production of recombinant proteins. Compared to other organisms, plants have several advantages for producing foreign proteins. They have the eukaryotic post-translational modification system and no risk of product contamination by mammalian viruses, pathogens and oncogenes (Ma et al. 2005). Plant production also may have a lower cost when field crops are used (Conley et al. 2011a). When transgenic crops are used as bioreactors for production of recombinant proteins, high levels of production are critical and desired for practical use (commercialization). To obtain high levels of accumulation of recombinant proteins, targeting proteins to sub-cellular locations optimal for their accumulation has proved quite successful. However, some problems remain in using transgenic plants for the production of recombinant proteins such as the instability of recombinant proteins in plants.

Recently, approaches involving tissue-specific transgene expression, organelle-specific protein targeting and stabilizing fusion partners in plant-based expression platforms have been proposed to improve the stability and yield of several proteins (Streatfield 2007; Benchabane et al. 2008). In practice, the use of several tissue-/organ-specific promoters (Potenza et al. 2004) or the addition of an N-terminal signal peptide sequence to a protein-coding transgene (Chrispeels and Faye 1996) have a strong impact on the yield and quality of recombinant proteins. In this study, the 22-kDa *alpha*-zein signal peptide (ZSP) from maize was used to increase the accumulation of recombinant CD14 protein. Zeins are a group of prolamin storage proteins that are synthesized in the endoplasmic reticulum (ER) where they form accretions called ER protein bodies in starchy endosperm cells (Pompa and Vitale 2006). In maize aleurone cells, synthesized zeins are accumulated inside protein storage vacuoles (PSVs) instead of the ER (Reyes et al. 2011). The entry of zeins into the ER occurs cotranslationally, and their signal peptide is cleaved from

the nascent polypeptide chain as it enters the luminal space of the ER. It is important to note that signal peptides are essential to direct zein proteins into the rough ER where protein body vesicles form and stable proteins accumulate (Randall et al. 2005). Because proteolytic degradation is a major problem in the aqueous environment of leafy crops (Benchabane et al. 2008), we hope to make use of the 22-kDa *alpha*-zein signal peptide (ZSP) to direct recombinant human CD14 protein into the ER. From the ER, CD14 will be secreted into the PSV, where the rCD14 protein can accumulate in large quantities and maintain its activity.

In this study we used a molecular recombination approach to improve the rCD14 levels in transgenic tobacco plants. The 22-kDa ZSP was used to direct the recombinant proteins to the ER. The production of bioactive rCD14 protein in transgenic tobacco leaves was significantly improved by using the 22-kDa ZSP. We found that 19.54 μg rCD14 protein g^{-1} tobacco leaf (fw) accumulated in the transgenic plants.

Materials and methods

Cloning the cDNAs encoding human CD14 and the 22-kDa ZSP

Based on the human CD14 coding sequence (GenBank accession no. NM_000591), primers CD-1 and CD-2 (Table 1) were designed and synthesized. Human mammary gland cDNA library (Clontech, USA) was used to amplify the CD14 encoding sequence and subsequently it was subcloned into the pGEM-T-easy vector (Promega, USA) before sequencing. Proper restriction sites (*Bam*HI and *Sac*I) were added to the 5' and 3' ends of the *CD14* coding region, and the signal peptide was omitted by amplification using primers CD-3 and CD-4 (Table 1). The PCR product named *no signal CD14* (*nsCD14*) was ligated into the pGEM-T-easy vector (Promega, USA) and sequenced.

Based on the 22 kDa *alpha*-zein coding sequence (GenBank accession no. NM_001112469), primers Z-1, Z-2 and Z-3 (Table 1) were designed. Because the signal peptide sequence is 63 bp in length, a fragment of the 22-kDa *alpha*-zein cDNA sequence including the signal peptide was first obtained by PCR using primers Z-1 and Z-3. Subsequently restriction sites for *Xba*I and *Bam*HI were added to the cDNA sequence encoding the 22-kDa ZSP by using the above PCR products as a template, and amplified with primers Z-1 and Z-2. The PCR-amplified fragments were ligated into the pGEM-T-easy vector (Promega, USA) and sequenced.

Table 1 Primers used for gene clone and for PCR and RT-PCR

	Primer sequence (5'–3')
PCR primer name	
CD-1	ATGGAGCGCGCTCTGCTTGTGCTG
CD-2	TTAGGCAAAGCCCCGGGCCCTTGAG
Z-1	<u>CTCTAGA</u> AATGGCTACCAAGATATTATCCC (<i>Xba</i> I site underlined)
Z-2	<u>AGGATCCC</u> GCATTTGTTGCGCTCACAA (<i>Bam</i> HI site underlined)
Z-3	TGTGATGGCTTGTATTGTTAGATGTGC
CD-3	<u>CGGGATCC</u> ACCACGCCAGAACCTTGTGA (<i>Bam</i> HI site underlined)
CD-4	<u>CGAGCTCT</u> TAGGCAAAGCCCCGGGCC (<i>Sac</i> I site underlined)
CD-5	CAACTTCTCCGAACCTCA
CD-6	GGCGTCTCCATTCCTGTGT
RNR ₂ -F	AGAAGAGCCTTTGCTGGCC
RNR ₂ -R	CCAACAATCTATCAGCCACG
CD-7	TGTGAGCTGGACGATGAAGA
CD-8	GCGAACGACAGATTGAGGG
ZSP- <i>Nco</i> I F	CATG <u>CCATGG</u> ACATGGCTACCAAGATATTATCCC (<i>Nco</i> I site underlined)
CD- <i>Nco</i> I F	CATG <u>CCATGG</u> ACATGGAGCGCGCTCTGCTT (<i>Nco</i> I site underlined)
CD- <i>Nco</i> I R	CATG <u>CCATGG</u> AGGCAAAGCCCCGGGCCCTT (<i>Nco</i> I site underlined)
RT-PCR primer name	
CD-RT-1	CGCAACACAGGAATGGAG
CD-RT-2	CGAACGACAGATTGAGGGA
Actin-1	CTATTCTCCGCTTTGGACTTGGCA
Actin-2	AGGACCTCAGGACAACGGAAACG

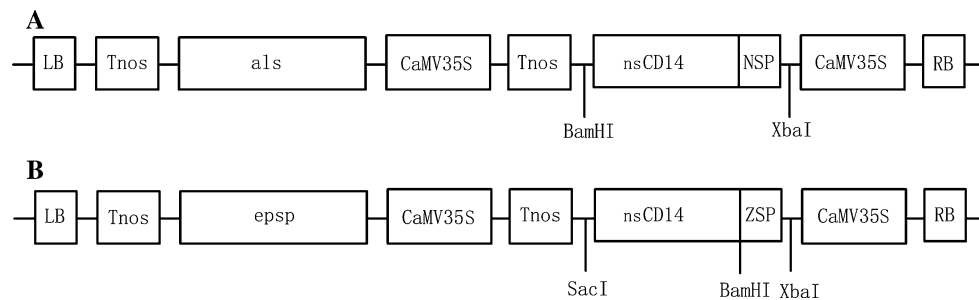


Fig. 1 CD14 expression constructs used for tobacco transformation. **a** The pCPA-NSP-nsCD14 construct. **b** The pCPE-ZSP-nsCD14 construct. 35S*CaMV* 35S promoter from cauliflower mosaic virus, NSP the native signal peptide sequence of *CD14* gene, ZSP the 22-kDa *alpha*-zein signal peptide sequence, nsCD14 human CD14

coding sequence lacking its own CD14 signal sequence, *Tnos* nos terminator, *als* a mutational chlorosulfuron-resistance acetolactate synthase gene from *Arabidopsis*, *epsp* a mutational glyphosate resistance 5-enolpyruvylshikimate-3-phosphate synthase gene from *E. coli*, LB left border, RB right border

Construction of plant expression vectors and tobacco transformation

The full-length *CD14* gene including its own native signal peptide sequence (NSP) and nsCD14 was ligated into the Ti plasmid pCAMBIA1300-P35sMCSnos-*als* (pCPA) to produce pCPA-NSP-nsCD14 (Fig. 1a). A *Bam*HI-*Sac*I-digested nsCD14 gene fragment was fused to an *Xba*I-*Bam*HI-digested 22-kDa ZSP coding fragment and ligated

into the *Xba*I-*Sac*I sites in the pCAMBIA1300-P35sMCSnos-*epsps* (pCPE) plasmid, to generate recombinant pCPE-ZSP-nsCD14 (Fig. 1b).

Plasmids pCPA-NSP-nsCD14 and pCPE-ZSP-nsCD14 were separately introduced into *A. tumefaciens* strain LBA4404 using the freeze-thaw method. After tobacco transformation using the leaf disc transformation method (Horsch et al. 1985), transformants with the construct pCPA-NSP-nsCD14 or pCPE-ZSP-nsCD14 were selected

on MS medium containing 25 mg l⁻¹ of the herbicide Lvhuanglong® (including 15 % chlorsulfuron, produced by the Shenyang Pesticide Company, Shenyang, China) or 8 mg l⁻¹ of the herbicide glyphosate (Monsanto, USA) and 500 mg l⁻¹ of cefotaxime. Resistant transformants were transplanted to soil in pots and self-pollinated to produce the next generation (T1) in a green house under standard conditions (16 h light/25 °C, 8 h dark/19 °C). A number of homozygous T1 transgenic plants from independent transformants were selected to produce the next generation. T2 transgenic plants were used for analysis.

PCR, reverse transcription (RT)-PCR, Western Blot analysis and ELISA

Genomic DNA was extracted from tobacco leaves of putative transgenic lines and WT lines using the cetyltrimethylammoniumbromide (CTAB) method. PCR analysis was performed with the specific primers CD-5 and CD-6 (Table 1) for confirmation of *CD14* gene in transformant tobaccos.

To check the level of transgene expression, total RNA was extracted from the young leaves of transgenic and WT tobacco using the Trizol reagent (Sangon, Shanghai, China) and cDNA synthesis was performed with an RT reagent kit (Takara, Dalian, China) according to the manufacturer's protocol. The primers for RT-PCR were CD-RT-1 and CD-RT-2 for the *CD14* cDNA, and Actin-1 and Actin-2 (Table 1) for the tobacco *actin* gene. Transgenic tobacco plants carrying single-copy transgene were selected using the method described by Kihara et al. (2006). In brief, we used two pairs of primers with the same T_m values. The primers CD-7 and CD-8 were used to amplify the *CD14* gene (Table 1). As a reference, the primers RNR₂-F and RNR₂-R were used to amplify a known single-copy gene in tobacco, *RNR₂* (ribonucleotide reductase R2 protein, GenBank accession no. X92443) (Table 1) (Chabouté et al. 1998). The PCR reaction parameters were one cycle at 94 °C for 3 min and 24 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min.

To estimate protein concentration in transgenic tobacco, tobacco leaves (1 g) were homogenized in 3 ml of ice-cold buffer containing 50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 0.1 % (V/V) Triton X-100, 1 % β-mercaptoethanol, pH 6.5. After incubation for 3–4 h, the extracts were centrifuged (13,000 g) for 15 min at 4 °C. Then the concentration of the total soluble protein in the supernatants was measured by the Bradford method (Bradford 1976) using a Modified Bradford Protein Assay Kit (Mutisciences Biotech Co., Ltd). For western blotting analysis, the protein extracts were denatured at 100 °C for 10 min in SDS-PAGE loading buffer, and separated on a

12 % acrylamide gel. Then the protein mix was transferred onto PVDF membrane by semi-dry electroblotting. The *CD14* protein was bound by a 1:400 dilution of rabbit anti-human *CD14* polyclonal antibody (Boster Company, China) and then detected with a goat anti-rabbit-alkaline phosphatase conjugated secondary antibody (Boster Company, China) at 1:5,000. Colorimetric detection was performed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Sangon, China) as a substrate. The protein marker (Amersham 17-0446-1) was purchased from Beijing Jingkehongda Biotechnology Co., Ltd (China). Recombinant Human *CD14* CHO derived (ProSpec, Israel) was used as a control in western blotting.

The concentration of *CD14* in the total soluble protein extracts was determined using an ELISA kit (ADL, USA) for total soluble Cluster of Differentiation 14. To perform ELISA experiment, 200 μg of total soluble protein was used for each sample and three biological replicates were performed for each sample. The concentration of *CD14* in the total soluble protein extracts was calculated by measuring the absorbency A₄₅₀ and comparing to a standard curve. Finally, the *CD14* content in tobacco leaves (fw) was calculated by multiplying the content of the total soluble protein in tobacco leaves (fw) by the concentration of *CD14* in the total soluble protein.

Biological activity of the recombinant human *CD14*

The LPS-binding activity of the recombinant human *CD14* was measured as described by Yin et al. (2002). U937 cells, from a human histiocytic lymphoma cell line (Hallbeck et al. 2001), were cultured in RPMI 1640 medium (Gibco, Invitrogen Corp.) supplemented with 10 % calf serum (Gibco, Invitrogen Corp.). Then the cells were collected and washed three times in RPMI 1640 medium with 5 % calf serum. The cells were counted and the cell concentration was adjusted to 1.0 × 10⁵ ml⁻¹. Meanwhile, the total soluble protein extracts from the leaves of each tobacco plant were filtered through the sterile 300 mesh nylon nets and its concentration was diluted to 15 μg ml⁻¹. The diluted protein extracts were incubated with the cells for 48 h in humidified air with 5 % CO₂ at 37 °C in the presence of 100 ng ml⁻¹ or 1 μg ml⁻¹ LPS (Sigma, St. Louis, USA). U937 cells cultured in the presence or absence of LPS were used as controls.

The U937 cells were harvested, and washed twice with ice-cold PBS containing 2 % calf serum. Then the cells were incubated with 20 μl Fluorescein isothiocyanate (FITC)-anti human *CD14* (eBioscience, San Diego, USA) for 30 min at 4 °C. Finally the cells were washed twice with ice-cold PBS and analyzed by flow cytometry using a FACScan (Epics-XL, Beckman Coulter).

Subcellular localization assay

To construct reporter constructs, the *CD14* gene was PCR-amplified from the construct pCPA-*NSP-nsCD14* with the primers CD-*NcoI* F and CD-*NcoI* R (Table 1). The amplified products were sequenced by Biosune Company (Biosune, Beijing, China). Confirmed fragments with no mutations were cloned into the *NcoI* site located upstream of the *gfp* reporter gene in the vector pCambia1302 (Cambia, Australia). This construct was named p35S::*CD14-GFP*. Meanwhile, the *ZSP-CD14* fusion gene was amplified from the construct pCPE-*ZSP-nsCD14* by PCR with the primers ZSP-*NcoI* F and CD-*NcoI* R (Table 1). After sequencing, the confirmed products without mutations were ligated into the pCambia1302 vector in using the method described above. The construct was named p35S::*ZSP-CD14-GFP*. These plasmids were then introduced into the *Agrobacterium tumefaciens* strain EHA 105. *Agrobacterium*-mediated transient expression assays were performed on the leaves of 6-week-old tobacco plants as previously described (Kapila et al. 1997; Yang et al. 2000). The infiltrated materials were maintained at 22 °C in the dark for 3 days, and then the leaves were sampled for the GFP fluorescence assay.

The laser scanning confocal microscopy (Leica, Germany) was carried out with a HCPL Fluotar 10×/0.3 NA objective lens. GFP was excited at 488 nm using an argon ion laser, and emission from 505 to 555 nm was detected.

Statistical analysis

All data were presented as mean ± standard deviation (SD). Comparisons between transgenic and WT plants or *CD14* and *ZSP-CD14* transgenic lines were performed using Student's *t* test. A *P* value <0.05 was considered to be statistically significant. All statistical analyses were done using SigmaPlot 10.0.

Results

Production and confirmation of transgenic tobacco plants

To express CD14 in tobacco, plant expression vectors pCPA-*CD14* and pCPE-*ZSP-CD14* were constructed and transformed into tobacco. The tobacco leaf discs from sterile seedlings were infected with *A. tumefaciens* harbouring pCPA-*CD14* or pCPE-*ZSP-CD14*, and plantlets were regenerated on the medium containing herbicide Lvhuanglong® or glyphosate. PCR assays for the transgenes in T0 and T1 plants were performed to identify transgenic ones.

Transgenic tobacco plants from the T2 generation were reconfirmed by PCR (Fig. 2a, b). Fragments of about 600 bp were amplified using primers CD-5 and CD-6 (Table 1) using the genomic DNA of transgenic tobacco plants as template. The phenotypes and growth rates of transgenic plants were similar to the WT plants. As the high-copy transgenes might interfere with gene expression, we selected transgenic tobacco lines with a single-copy transgene to perform the experimental analyses (López et al. 2010; Mubmann et al. 2011; Wen et al. 2012; Pires et al. 2012). Finally, eight independent homozygous, single-copy *CD14* transgenic lines and ten independent homozygous, single-copy *ZSP-CD14* transgenic plants were selected from T2 transgenic tobacco plants by using the method from Kihara et al. (2006).

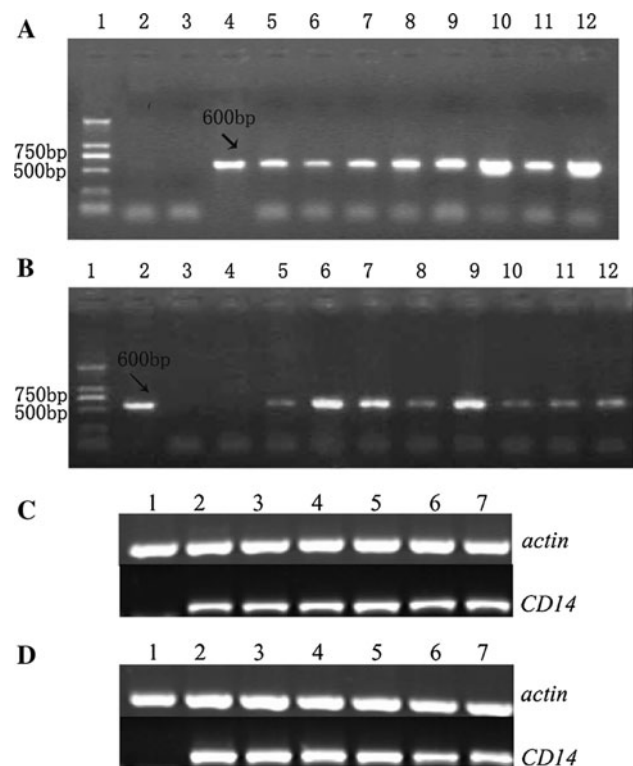


Fig. 2 Characterization of transgene transcription in transgenic tobacco plants. **a** PCR analysis of transformed tobacco expressing the *CD14* cDNA. Lane 1 DL2000 marker, Lane 2 WT tobacco as a negative control, Lane 3 H₂O, Lane 4 pCPA-*CD14* plasmid as a positive control, Lanes 5–12 transgenic tobacco lines CD-1–CD-8. **b** PCR analysis of transformed tobacco expressing the *ZSP-CD14* fusion cDNA. Lane 1 DL2000 marker, Lane 2 pCPE-*ZSP-CD14* plasmid as a positive control, Lane 3 WT tobacco as a negative control, Lane 4 H₂O, Lanes 5–12 transgenic tobacco lines ZCD-1–ZCD-8. **c** RT-PCR detection of *CD14* expression in transgenic *CD14* tobacco plants. Lane 1 WT tobacco as a negative control, Lanes 2–7 transgenic tobacco lines CD-1 and CD-3–CD-7. **d** RT-PCR detection of *CD14* expression in transgenic *ZSP-CD14* tobacco plants. Lane 1 WT tobacco as a negative control, Lanes 2–7 transgenic tobacco lines ZCD-1–ZCD-6

CD14 expression was increased in the *ZSP-CD14* transgenic tobacco

RT-PCR, western blotting analysis and ELISA were performed to measure the level of expression of CD14 in the T2 single-copy transgenic tobacco plants. RT-PCR demonstrated that CD14 was expressed in the transgenic tobacco plants (Fig. 2c, d). Specific PCR products of about 170 bp were detected in all six *CD14* transgenic lines and the *ZSP-CD14* transgenic lines. A 380 bp *actin* fragment was amplified by RT-PCR as an internal control. There was no expression of CD14 in WT plants. To further measure CD14 expression and confirm the RT-PCR results, western blotting was performed using the total soluble protein extracted from both transgenic and WT leaves. The concentration of the total soluble protein was about $1\text{--}1.5 \mu\text{g} \mu\text{l}^{-1}$ in extracted solution, and 100 μg of total soluble protein for each sample was used for SDS-PAGE. The result of western blotting showed that, a ~ 48 kDa band was detected both in the *CD14* transgenic lines and the *ZSP-CD14* transgenic lines (Fig. 3a, b). The CD14 protein was not detected in the WT tobacco plants. It should be noted that there were differences in expression levels between the different transgenic lines (Fig. 3a, b).

In plants that were positive for the rCD14 protein by western blot, the rCD14 concentration was measured by ELISA (Fig. 3c). The average concentration of the rCD14 protein in the *CD14* transgenic lines was 0.21 ± 0.03 % of total soluble protein, which corresponds to $7.22 \pm 1.04 \mu\text{g} \text{g}^{-1}$ tobacco leaves (fw), calculated as described in Materials and Methods. The highest concentration reached 0.26 % of total soluble protein, corresponding to $8.47 \mu\text{g} \text{g}^{-1}$ tobacco leaves (fw). In the *ZSP-CD14* transgenic tobacco plants, the average concentration of the rCD14 protein was 0.46 ± 0.07 % of total soluble protein [$16.88 \pm 2.39 \mu\text{g} \text{g}^{-1}$ tobacco leaves (fw)], and the highest concentration was 0.60 % of total soluble protein, corresponding to $19.54 \mu\text{g} \text{g}^{-1}$ tobacco leaf (fw) (Fig. 3c). These results suggested the zein signal peptide indeed increased the level of accumulation of the rCD14 protein in the tobacco leaves.

ZSP-CD14 transgenic plants produced rCD14 protein with LPS binding activity

To assess the bioactivity of the rCD14 protein produced by tobacco leaves, LPS-binding activity of the rCD14 protein was analyzed in vitro using flow cytometry (FMC). The FMC assay is based on the theory of mCD14 from monocytes binding to anti-CD14 mAb. The amount of cell-associated FITC-anti human CD14 mAb is determined by FMC. With a certain amount of LPS stimulation, changes in the expression level of mCD14 on the surface of U937

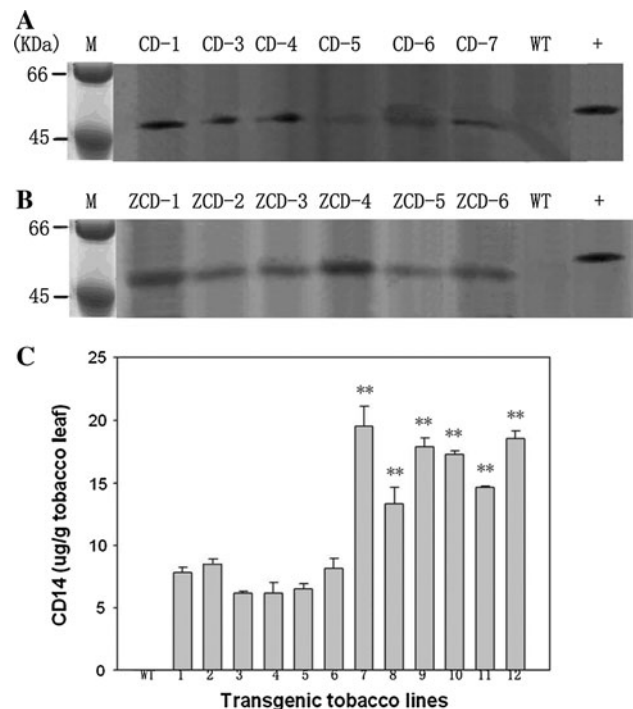


Fig. 3 Western blot detection of rCD14 in transgenic tobacco and quantification of the rCD14 protein in transgenic tobacco leaves. **a** Western blot detection of rCD14 in transgenic *CD14* tobacco. The soluble protein extracts from six transgenic *CD14* tobacco lines were subjected to western blotting with rabbit anti-human CD14 polyclonal antibody followed by a goat anti-rabbit-alkaline phosphatase conjugated secondary antibody. **b** Western blot detection of rCD14 in transgenic *ZSP-CD14* tobacco. The soluble protein extracts from six transgenic *ZSP-CD14* tobacco lines were subjected to western blotting. The WT plant was used as a negative control. **M** protein marker. **+**, recombinant human CD14 CHO derived was used as a positive control. **c** Quantification of the rCD14 protein in transgenic tobacco leaves. An ELISA assay was performed to measure the amount of rCD14 protein in the leaves of transgenic *CD14* tobacco lines (1–6) and transgenic *ZSP-CD14* tobacco lines (7–12). 1–6 Transgenic *CD14* tobacco lines CD-1 and CD-3–CD-7. The rCD14 contents in the six *CD14* transgenic lines were respectively 0.21, 0.26, 0.18, 0.19, 0.21 and 0.24 % of total soluble protein calculated by the ELISA standard curve. The rCD14 expression levels in $\mu\text{g} \text{g}^{-1}$ of leaf (fw) can be given by the content of the total soluble protein in tobacco leaves (fw) multiplied by the concentration of CD14 in the total soluble protein. 7–12 Transgenic tobacco lines ZCD-1–ZCD-6. The rCD14 contents in the six *ZSP-CD14* transgenic lines were respectively 0.60, 0.41, 0.42, 0.41, 0.44 and 0.50 % of total soluble protein calculated by the ELISA standard curve. The rCD14 expression levels in $\mu\text{g} \text{g}^{-1}$ of leaf (fw) can be given as described above. Extracts of soluble proteins from the leaves of WT tobacco plants were used as a control. Values are the mean \pm standard error of three biological samples. Significant difference between CD14 transgenic lines and ZSP-CD14 transgenic lines was confirmed by Student's *t* test (** $P < 0.01$)

cells can be measured based on the level of change in cell-associated mAb, which is measured using FMC. In this experiment, when the concentration of LPS was between 100 ng ml^{-1} and $1 \mu\text{g ml}^{-1}$, the cultured U937 cells proliferated normally. Cells were incubated with or without

plant-derived rCD14 for 48 h. Then, the effects of the plant-produced rCD14 incubated with LPS on the levels of mCD14 expression were measured with a FITC-anti human IgG conjugation. The difference in the amount of positive cells was measured by FMC between 100 ng ml⁻¹ LPS stimulation (positive cells, 92.2 %) and 1 µg ml⁻¹ LPS stimulation (positive cells, 95.1 %) and the difference was not significant. Therefore, only the cells stimulated with 100 ng ml⁻¹ LPS were used in the following FMC experiment.

Compared to the protein extracts from WT plant, treatment of cells with protein extracts (15 µg ml⁻¹) from *CD14* or *ZSP-CD14* transgenic lines and LPS to the cells led to a significant decrease in the amount of mCD14 measured by FMC. The proportion of positive cells for the group with rCD14-3 was 78.95 ± 3.16 %, whereas the proportions of positive cells for the three groups with ZSPrCD14-1, ZSPrCD14-3 and ZSPrCD14-6 were 64.84 ± 2.62 %, 69.31 ± 2.96 % and 67.23 ± 1.89 % (Fig. 4). The competitive binding of LPS to the plant rCD14 and mCD14 on the surface of U937 cells led to a decrease of the density of systematic free LPS. Because of the decrease in the density of systematic free LPS, the effects of LPS stimulation on the cells became weak, which inhibited U937 cells in their production of mCD14. In addition, it was indicated that higher rCD14 protein content in protein extracts from *ZSP-CD14* transgenic tobacco resulted in lower proportions of positive cells measured in the LPS-binding activity experiment compared to that from *CD14* transgenic tobacco (Fig. 4). The results indicated that the rCD14 protein from transgenic *ZSP-CD14* tobacco had similar LPS-binding activity to the native CD14 protein.

Agrobacterium-mediated GFP transient expression assay in tobacco leaves

To elucidate the targeting properties of the native signal peptide of CD14 and *alpha*-zein signal peptide, the sub-cellular localization of visual marker GFP fused to CD14 or *ZSP-CD14* was examined in tobacco leaves by the method of *Agrobacterium*-mediated transient expression assay. Leaves infiltrated with the p35S::*CD14-GFP* construct had low levels of the rCD14 protein distributed in the cytoplasm of tobacco mesophyll cells (Fig. 5b). The leaves infiltrated with the p35S::*ZSP-CD14-GFP* construct had a higher accumulation level of the rCD14 protein in PSVs of mesophyll cells (Fig. 5c). The construct pCAMBIA1302 was also used in this experiment as a control. Leaves infiltrated with pCAMBIA1302 had a low level of accumulation of GFP protein in the cytoplasm of tobacco mesophyll cells (Fig. 5a). The assay results suggested the *ZSP* could direct the rCD14 protein to the ER where the

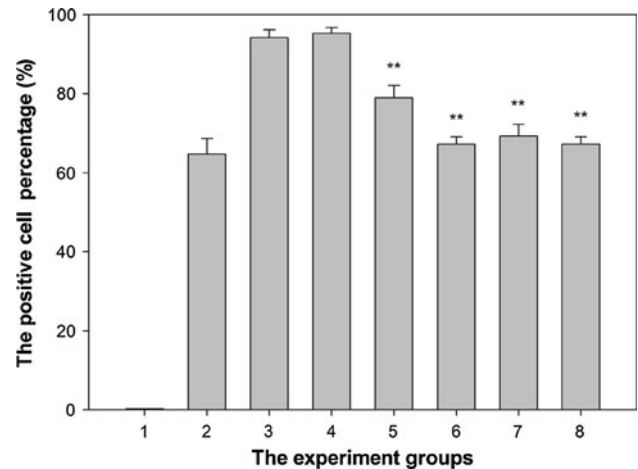


Fig. 4 The LPS-binding activity assay of the rCD14 protein. 1 The group (U937 cells), was the background. 2 The group (U937 cells + anti-CD14), was the control. 3 The group (U937 cells + anti-CD14 + LPS) was the control. 4 The group (U937 cell + anti-CD14 + LPS + WT). Protein extracts from WT leaves (WT) were added to the U937 cells (containing 100 ng ml⁻¹ LPS) before addition of FITC-anti-CD14 monoclonal antibody (anti-CD14). The purpose of the group is set to eliminate the impact of protein extracts from tobacco leaves on the LPS-binding reaction when analyzing the LPS-binding activity of rCD14 protein. 5 The group (U937 cell + anti-CD14 + LPS + rCD14-3). Protein extracts from leaves of transgenic *CD14* tobacco line CD-3 were added to the U937 cells (containing 100 ng ml⁻¹ LPS) before addition of FITC-anti-CD14 monoclonal antibody (anti-CD14). 6–8 The group (U937 cell + anti-CD14 + LPS + ZSPrCD14-1/3/6). Protein extracts from leaves of three transgenic *ZSP-CD14* tobacco lines ZCD-1, ZCD-3 and ZCD-6 were separately added to the U937 cells (containing 100 ng ml⁻¹ LPS) before addition of FITC-anti-CD14 monoclonal antibody (anti-CD14). The amount of positive U937 cells associated anti-CD14 was measured by FMC. The proportion of positive cells indirectly reflects the LPS-binding activity of the rCD14 protein. The differences between WT tobacco and transgenic tobacco line were significant with ***P* < 0.01 using the *t* test. Values are the mean ± standard error of three biological samples

recombinant proteins were secreted into PSVs and accumulated.

Discussion

Currently, more and more attention is being given to the study of CD14 because of its broad application potential in preventing infection, improving immunity and treating disease. Several strategies have been used to produce sCD14 in *E. coli*, yeast, mammalian cells and tobacco (Majerle et al. 1999; Yin et al. 2002; Nomura et al. 2003; Blais and Altosaar 2006). Compared to microbial and mammalian cell culture systems, plant bioreactors have advantages for gene expression and protein production. However, low levels of accumulation and protein instability have been noted as limiting factors for the commercial application of transgenic plants. Improvements

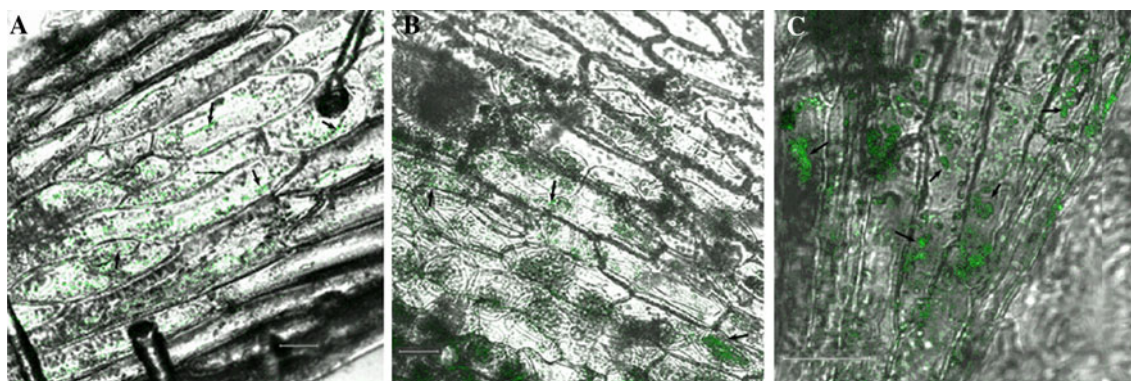


Fig. 5 Subcellular localization of CD14-GFP proteins in tobacco leaves. **a** The mesophyll cells of tobacco leaves infiltrated with the pCAMBIA1302 construct; **b** the mesophyll cells of tobacco leaves

infiltrated with the p35S::CD14-GFP construct; **c** the mesophyll cells of tobacco leaves infiltrated with the p35S::ZSP-CD14-GFP construct

have been achieved by boosting transcription, translation and stability of transcripts or translated products (Streatfield 2007; Takaiwa et al. 2007; Torrent et al. 2009).

To enhance the yield of recombinant protein in this study the 22-kDa zein signal peptide from maize was used to replace the native signal peptide of the human CD14 gene. Tobacco was transformed with the CD14 gene containing either the native SP or the 22-kDa ZSP by *Agrobacterium*-mediated transformation to produce stable transgenic plants. Western blotting and ELISA showed that tagging rCD14 with the 22-kDa ZSP enhanced expression. The recombinant protein was also found to have good LPS-binding activity. *Agrobacterium*-mediated GFP expression transient assays suggested that the ZSP could direct the recombinant proteins to the ER where they were secreted and accumulated in the PSVs of mesophyll cell. These results demonstrated that the human CD14 gene was successfully expressed in the leaves of transgenic tobacco with high levels of protein accumulation and normal protein activity.

Commercial CHO human CD14 has a molecular weight of 50 kDa. In both the transgenic ZSP-CD14 tobacco and transgenic CD14 tobacco, the recombinant CD14 proteins had molecular masses of about 48 kDa as measured by western blotting. In a report by Blais et al. (2006) the rCD14 protein produced by transgenic tobacco seeds had a molecular weight of 46 kDa. They inferred that the differences in observed molecular weight between the rCD14 from transgenic plants and the commercially available CHO CD14 could be attributed to different post-translational modifications, such as glycosylation, in plant and mammalian cells (Blais and Altosaar 2006). In addition, the differences in the molecular masses of the rCD14 proteins in different transgenic tobacco lines (Girard et al. 2004; Blais and Altosaar 2006) could be explained by the use of different organs and signal peptides for transgene expression. These differences could lead to variations in

the sub-cellular localization of the recombinant protein, which in turn could result in different post-translational modifications (Wright et al. 2001). In previous studies, the glycosylated state of CD14 was found to contribute to differences in the observed molecular mass of the rCD14 proteins when expressed in different organisms; however, differences in glycosylation did not seem to affect the ability of the protein to bind to LPS (Stelter et al. 1996). Therefore, we speculate that glycosylation does not influence the LPS-binding activity of rCD14.

An ELISA assay was performed to investigate if attaching the signal peptide from maize zein enhanced the levels of accumulation of the rCD14 protein in the transgenic tobacco. The results showed that the protein level of rCD14 in ZSP-CD14 transgenic tobacco was increased to $19.54 \mu\text{g g}^{-1}$ of tobacco leaf (0.6 % of the leaf total soluble protein) and was significantly higher than the protein level in transgenic CD14 tobacco ($8.47 \mu\text{g g}^{-1}$ of tobacco leaf).

We concluded that using the 22-kDa *alpha*-zein ZSP increased the accumulation of the rCD14 protein in transgenic tobacco leaves. Tobacco has many desirable agronomic attributes, such as high biomass yields (more than $100,000 \text{ kg ha}^{-1}$) and high soluble protein levels (Conley et al. 2011b). Most importantly, tobacco is a non-food, non-feed crop, which minimizes regulatory barriers by eliminating the risk of plant-made recombinant proteins entering the food supply (Menassa et al. 2001; Conley et al. 2011b). Previous studies have expressed the rCD14 protein in tobacco seed endosperm and reached a concentration of $16 \mu\text{g g}^{-1}$ of seeds (Blais et al. 2006). However, if the expression platform is based on leaves, its advantages are obvious. One advantage is that flowering is not needed, which could reduce the possibility of gene leakage into the environment through pollen or seed dispersal (Twyman et al. 2003). Another advantage is that the rCD14 protein can be obtained quickly and continuously from fully expanded green tobacco leaves that are rich in soluble

proteins, which is very important for the downstream production process. Therefore, the improved expression of rCD14 accumulated in tobacco leaves may offer a low-cost, safe and sufficient supply of this immune protein for practical use.

The accumulation of the rCD14 protein in this study has been efficiently improved through localization of the protein within the transgenic plant cells. This may lead to reduced degradation and more efficient storage of protein; however, unintended proteolysis of the recombinant protein expressed in leaf tissue has been reported in previous studies (Benchabane et al. 2008). In addition, the heterologous expression of the human CD14 protein in tobacco did not affect the normal growth and development of plants. Our results suggested that maize 22-kDa *alpha*-zein signal peptide could be used to increase the accumulation of recombinant protein in a heterologous environment.

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