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Plant expression of chicken secretory antibodies derived from combinatorial libraries

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Abstract

Delivery of secretory IgA antibodies (sIgA) to mucosal surfaces is a promising strategy to passively prevent infectious diseases. Plants have been proposed as biofactories for such complex immunoglobulin molecules. Recently, the molecular characterization of all four monomers of chicken sIgA (IgA immunoglobulin heavy and light chains, J-chain and secretory component) has been completed, allowing recombinant, up scaled production of chicken sIgA and extension of passive immune strategies to poultry. To test the suitability of the plant cell factory for bulk production of chicken sIgA, we studied the expression of chicken IgA, dIgA and sIgA *in planta*. To that end, new cassettes were designed that allowed the grafting of immunoglobulin variable regions derived from combinatorial libraries into full-size chicken IgA frames ready for plant expression. Using this system, 10 individual phage display clones, which had previously been selected against *Eimeria acervulina* antigens, were transferred "from phage to plant". Plant-made chicken antibodies showed strong differences in expression levels, which seemed governed mainly by the stability of their respective light chains. Finally, with the co-expression of chicken IgA heavy and light chains, J-chain and secretory component in *N. benthamiana* leaves we showed that plant cells are suitable biofactories for the production of assembled chicken sIgA complexes.

Keywords: Molecular farming; Recombinant antibodies; Oral immunotherapy; Chicken; Secretory IgA

1. Introduction

The threat posed by emerging zoonoses and the need to reduce the use of antibiotics in the food chain demand the development of alternative methods to protect livestock. Antibodies administered by the oral route are known to efficiently protect mucosal surfaces against

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pathogenic infections (Offit and Clark, 1985; Winner et al., 1991; Czinn et al., 1993; Enriquez and Riggs, 1998), therefore oral passive immunotherapy represents a promising alternative for the use of antibiotics. In mammals, the most important isotype for mucosal protection is secretory immunoglobulin A (sIgA). Secretory IgA is formed in two stages: B-lymphocytes produce dimeric IgA molecules in the lamina propria. Dimerization is regulated by the J-chain. IgA dimers (dIgAs) bind to the polymeric immunoglobulin receptor (pIgR) at the basolateral surface of mucosal epithelium cells and are subsequently transcytosed to the intestinal lumen. There, the extracellular portion of pIgR, known as secretory component (SC), is proteolytically cleaved and remains attached to the IgA polymer rendering the heteromultimeric structure known as secretory IgA (Mostov and Kaetzel, 1999). Secretory IgA constitutes a highly stable complex that covers mucosal surfaces, acting as a first barrier against infection (Phalipon and Corthesy, 2003).

Several indications suggest that sIgA-based oral passive immunotherapy can also be applied for prophylaxis against pathogens in birds. The presence of protective antibodies from the alpha isotype in chicken mucosal surfaces is known for long (Mansikka, 1992). A cDNA encoding a J-chain homologue has been reported (Takahashi et al., 2000). More recently, the presence of sIgA in birds was confirmed with the cloning and characterization of a chicken homologue of the mammalian *pIgR*. Chicken pIgR protein was shown to associate with IgA, forming high molecular weight complexes that resemble the mammalian sIgA (Wieland et al., 2004). Structurally, chicken IgA differs significantly from mammalian IgA: it contains four instead of three constant domains and lacks the typical hinge region of mammalian alpha heavy chains (Mansikka, 1992). Also the chicken pIgR is different from its mammalian counterparts, as it lacks one additional immunoglobulin-like domain in its extracellular region (Wieland et al., 2004).

Bulk production of sIgA for passive immunotherapy requires efficient, low-cost manufacturing systems. Plants have been proposed as biofactories for recombinant antibodies as they offer considerable advantages in comparison to other organisms. Plant cells can express mammalian antibodies in several formats, from scFv to Fab and full-size IgG (Stoger et al., 2002). Ma et al. (1995) demonstrated that plant cells could assemble mammalian antibodies in a secretory form: tobacco plants transformed with all four genes encoding chimeric sIgA/G produced fully functional secretory antibodies. The secretory version of this antibody accumulated at high levels in the plant, retained specificity against *Streptococcus mutans* and conferred protective activity against dental caries (Ma et al., 1998). The high accumulation of sIgA/G in plants seems related with its retention in the endomembrane system (Frigerio et al., 2000; Wycoff, 2005). Assembled sIgA/G has also been produced in rice endosperm where it accumulates predominantly in vacuolar-derived protein bodies (Nicholson et al., 2005). It has to be noted that *in planta* assembly of sIgA is a rather artificial process, since in animal cells assembly between dIgA and SC (pIgR) takes place in the extracellular space as a part of the dIgA endocytosis process.

In this paper we report the ability of plants to express and assemble avian antibodies as a pre-requisite for extending sIgA-based passive immunotherapy to poultry. Previously, chicken phage display combinatorial libraries in Fab format were generated for the selection of antibodies against protozoa of the genus *Eimeria*, the causal agent of coccidiosis, an intestinal disease that causes substantial economical losses in poultry industry (Williams, 1999). The display and selection of antibody libraries was described elsewhere and resulted in a number of Fab clones showing similar binding activities against *Eimeria* complex antigenic mixes (Wieland et al., 2006). As an additional selection step prior to bulk production in animal feed plants, we decided to compare the *in planta* expression of several clones using a transient expression assay. Here we describe the conversion of 10 anti-*Eimeria* Fabs into full-size IgAs using newly designed plant expression cassettes, and their subsequent expression in *N. benthamiana* leaves by agroinfiltration. The use of this transient expression system as opposed to stable transformation allowed us to investigate the intrinsic expression characteristics without interference of positional effects. We found drastic differences in the expression levels of the different antibody idiotypes, highlighting the convenience of introducing an *in planta* selection step for plant-made phage display-derived antibodies. Finally, we demonstrate that the co-expression of the four components of the chicken sIgA can render assembled sIgA

complexes, opening the way for the extension of sIgA-based passive immunotherapy to poultry.

2. Materials and methods

2.1. Phage display vectors and antibody selection

The display and selection of chicken antibodies using a phage display approach is described elsewhere (Wieland et al., 2006). Briefly, a new phagemid vector, pChick3, was constructed by incorporating chicken specific sequences into a bi-cistronic operon. pChick3 displays chicken antibodies as Fab fragments with the first constant domain of the alpha heavy chain fused to the capsid protein pIII of M13 phage. A combinatorial antibody library was constructed by cloning antibody cDNA isolated from lymphoid tissues of *Eimeria acervulina* infected chickens into pChick3, to obtain a library (Eckα1) with 1.2×10^7 individual transformants. Successive selection steps against complex *E. acervulina* antigens enriched the library for anti-*E. acervulina* binding antibody fragments. Ten of these antibody fragments, showing detectable binding activity to *E. acervulina*, were selected for full length expression and characterization *in planta*.

2.2. Construction of plant expression cassettes

The pRAPIg vectors were derived from pUCAP (van Engelen et al., 1995) with added mouse kappa light chain signal sequence for secretion (van Engelen et al., 1994). In the pRAPIgL version, phage display-derived chicken IgL sequences were incorporated as *Sal*I/*Xba*I fragments into the vector frame. For the construction of pRAPIgH, a DNA fragment (Cα1–4) encoding the four constant domains of chicken alpha heavy chain was amplified by PCR using forward primer 5′CCGAGCTCCGCCTCCGCCAGCCGCC3′ (02feb15) and reverse primer 5′CGCCGACGTCACC-TGTTATTAATCTAGAGG3′ (02feb14) from chicken bursa of Fabricius cDNA. The fragment was cloned in the pRAP frame by *Sst*I/*Xba*I digestion, resulting into pRAPCα1–4, which can incorporate VH as *Sal*I/*Sst*I fragments.

For the cloning of phage display-derived chicken sequences into pRAPIg vectors, individual Eckα1 library clones were used as a template in two separate PCR reactions of 10 cycles. Flanking *Sal*I/*Xba*I or *Sal*I/*Sst*I restriction sites were incorporated using forward primer 5′GGGTCGACG-GTTCCCTGGTGCAGGCAGCGCTGACTCAGCC3′ (02feb18) and reverse primer 5′CCTGAAGAGGTCC-GAGTGCTAATAGTCTAGACC3′ (02feb17) to amplify IgL and primers 5′CCGTCGACGGCGTGAC-GTTGGACGAGTCC3′ (02feb16, forward) and 5′CCACGGGACCGAAGTCATCGTGAGCTCCGC-CTCCGCCACCCG3′ (02jan06, reverse) for VH amplification. PCR-amplified bands were purified and ligated into pRAPIg vectors. The sequences of all PCR-derived constructs were verified by two-strand sequencing to avoid PCR-born errors. A schematic overview of the cloning procedure is provided in Fig. 1.

The expression cassette for chicken secretory component was based on the GG-pIgR cDNA

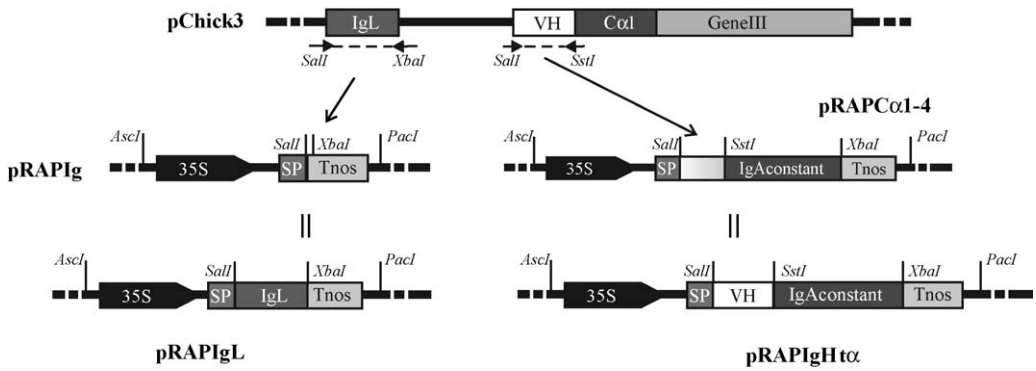

Fig. 1. "Phage to plant" expression system. Schematic overview for the cloning of the light (IgL) and α heavy chain (IgHα) in plant expression cassettes. SP, mouse κ signal peptide; 35S, CaMV35S promoter; Tnos, nopaline synthase terminator.

described by Wieland et al. (2004). A clone coding for SC, based in GG-pIgR but excluding the signal peptide and the transmembrane and cytoplasmic domains, was amplified by forward primer 5′CCCAGAGTCGACTTAAACCCAGTGTGTGGACCGCAG3′ (SC1) and reverse primer 5′GAGGCCGTTCCGCAGGCAGAGCCACCCGGGCTGGGG3′ (SC2) and cloned into pBAD/Thio-TOPO vector (Invitrogen, Belgium). This construct was used as template for a second PCR, using the same forward primer and reverse primer 5′CATCATCACCATCACCATTGATCTAGAACGGTCTCC3′ (BadR1). The product was transferred to pRAPIgL vector using *Sal*I and *Xba*I restriction sites. A plant expression vector for chicken J-Chain expression was constructed in a similar way as SC but conserving its endogenous signal peptide by using forward primer 5′GAAGGCACCATGGCGAGCTCTTTGCCGTGGGTGGCTTTG3′ (J1) and reverse primers 5′CCAACATCCTGCTATGCCGAATAGCCCGGGCCACTG3′ (J2).

For *Agrobacterium*-mediated expression, pRAP vectors were digested with *Asc*I/*Pac*I and the resulting inserts carrying the immunoglobulin chain, secretory component or J-chain expression cassettes were isolated and ligated into pBIN+ vector (van Engelen et al., 1995).

2.3. Agroinfiltration experiments

For plant expression of chicken immunoglobulin chains, SC, J-chain or YFP, pBIN+-derived vectors were transferred to *Agrobacterium tumefaciens* MOG101 strain. The transformed *Agrobacterium* cultures were grown as described (Kapila et al., 1997). Co-infiltrations were performed by mixing equal volumes of 1.0 $OD_{600\,nm}$ cultures of the involved bacteria, unless stated otherwise. A culture carrying pBIN+ vector without an insert was included to equalize bacterial concentrations when required. For all infiltration experiments we used 5-week-old *Nicotiana benthamiana* plants that were grown under standard conditions in a greenhouse. From every plant the second and third leaves from the top were used for expression studies. Infiltrated areas were marked and harvested after 4 days. Leaf material from one plant was pooled and immediately frozen in liquid nitrogen. Soluble protein was extracted by grinding the frozen leaf material using a mortar and pestle and subsequent addition of ice-cold PBS-T (PBS with 0.05% Tween-20) containing 1 mM Pefablock (Boehringer Mannheim, Germany). Cell debris was removed by centrifugation in an Eppendorf centrifuge (5 min/13,000 g/4 °C). Supernatant was stored at −80 °C for further analyses.

2.4. Western blotting and ELISA

For the detection of individual antibody chains or assembled IgA complexes, protein extracts of plant samples were mixed 1:1 with sample buffer (50 mM Tris–HCl pH 6.8; 2% (w/v) SDS; 10% (w/v) glycerol; 0.01% bromophenolblue, with or without addition of 40 mM DTT). SDS-PAGE was carried out using 7.5% polyacrylamide gels, except for the analysis of secretory IgA complexes, where Novex 3–8% Tris-acetate gels (Invitrogen) were used. After electrophoresis, the proteins were transferred to nitrocellulose membranes (Millipore, The Netherlands) by semi-dry blotting according to standard procedures. For all Western analyses, equal loading of proteins was confirmed by coomassie brilliant blue staining of a second SDS-PAGE gel. The membranes were blocked with PBS containing 0.05% Tween-20 (PBS-T) and 5% low fat milk powder. The immunoglobulin heavy and light chains were visualized by incubating the blots with goat anti-chicken IgA-peroxidase antibodies or with peroxidase-labelled goat anti-chicken light chain antibodies (both 1:7500; Bethyl, Montgomery, Texas). The reaction with ECL substrate (Amersham Biosciences, The Netherlands) was visualized by exposure to X-ray film.

The concentration of plant-produced IgA was determined by double sandwich ELISA. Microtitre plates were coated over night with anti-chicken IgA (1:2000; Bethyl) in carbonate buffer (1:5; 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Non-specific binding sites were blocked with 5% low fat milk powder in PBS-T. For determination of IgA concentration, standard chicken serum with a known IgA concentration was used (Bethyl). Dilutions of crude plant extracts and reference serum were made in PBS-T. After incubation for 1 h at room temperature, the amount of plant-produced antibodies was determined with peroxidase-labelled goat anti-chicken light chain antibodies (1:5000; Bethyl) and developed with ABTS (Amersham). Reactions were carried out for 30 min at room temperature. Concentrations of IgA in the plant

extracts were calculated by non-linear regression using the GraphPad PRISM 4.0 algorithm. Specific binding of plant-produced immunoglobulins was detected following a similar protocol. Plates were coated with *E. acervulina* antigens prepared as described (Wieland et al., 2006) and incubated with crude plant extracts for 1 h at 37 °C. The amount of bound plant-produced antibodies was determined with peroxidase-labelled goat anti-chicken IgA antibodies (1:5000; Bethyl). ABTS development was monitored after 3 h. After protein extraction from the leaves, total soluble protein concentration was determined by BCA protein assay kit (Pierce, Rockford, USA) using BSA-solutions as standard reference.

2.5. Northern blotting

RNA was isolated from leaves co-infiltrated with constructs for heavy and light chain expression at 4 days post-infiltration. Total RNA was isolated using the RNeasy Plant Mini kit including an on-column DNAse digestion with RNAse-free DNAse (both obtained from Qiagen, Leusden, The Netherlands). RNA concentrations were equalized by $OD_{260\,nm}$ measurements and verified by ethidium bromide staining of the gel. Ten micrograms of RNA per sample was blotted onto nylon membranes (HybondN+, Amersham) and hybridised with radio-labelled probes (^{32}P-dCTP) according to standard procedures. Both heavy and light chain probes were located in the constant domains of the immunoglobulin chains. DNA for the probes was obtained by digestion of pRAP IgHα vector with *Kpn*I and *Not*I, releasing an insert of 543 bp and digestion of pRAPIgL with *Avr*II and *Xba*I, resulting in a 261 bp insert.

3. Results and discussion

3.1. From phage display to plant expression

For the production of full-size chicken IgA in plants, pRAP expression cassettes (pRAPIgL and pRAP IgHα) were constructed (Fig. 1). The vector pRAPIgL was designed to function as an expression cassette for immunoglobulin light chains (IgLs). It comprises a CaMV35S promoter region followed by the signal peptide of the murine kappa light chain, a multiple cloning site, and the NOS terminator. Similarly, pRAP IgHα incorporates the same promoter region and signal peptide, as well as the four constant domains of chicken heavy chain of the alpha isotype (Cα1–4). Both vectors allow rapid transfer of antibody variable regions, which facilitates the cloning of sequences derived from combinatorial libraries.

Antibody variable domains were obtained from a previously developed chicken antibody library in Fab format (Wieland et al., 2006). The library was enriched for anti-*Eimeria* clones by selection against *E. acervulina* antigen mixes. The use of complex antigens in the selection procedure was aimed at the generation of a polyclonal antibody library (Sharon et al., 2000), ensuring a broad range of anti-*Eimeria* activity. In order to perform functional plant expression studies, we chose 10 individual Fab clones that showed detectable binding activity in ELISA assays against *E. acervulina* crude antigen preparation. Heavy (VH) and light (VL) variable regions from all 10 clones were subcloned into the corresponding pRAP expression cassette and transferred to the pBIN+ vector for plant expression. Co-expression of heavy (IgHα) and light (IgL) chains was obtained by agroinfiltration of equal volumes of *Agrobacterium* cultures carrying pRAPIgL and pRAP IgHα expression cassettes, respectively into *N. benthamiana* leaves.

3.2. Individual chicken antibodies show strong differences in their expression levels

Several infiltration experiments were performed, and although *in planta* IgA production levels were found to be influenced by physiological conditions (leaf age, incubation time, temperature), the relative accumulation levels of the different antibodies remained constant, independent of the greenhouse and assay conditions (data not shown). The results of a representative experiment are shown in Fig. 2. The functionality of the plant-made antibodies was analyzed by an *E. acervulina*-specific ELISA assay (Fig. 2A). In parallel, plant-produced IgA yields were quantified by sandwich ELISA (Fig. 2B). As can be observed in Fig. 2A, specific anti-*Eimeria* binding activity was detected directly in crude extracts from agroinfiltrated plants using the same *E. acervulina* antigen mixes employed during phage display selection, indicating that antibody functionality was retained *in planta*. Among the

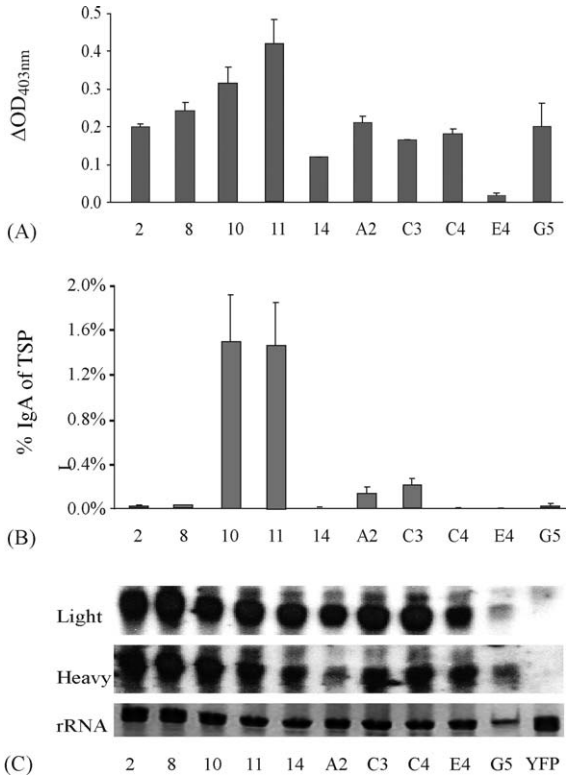

Fig. 2. Behaviour of chicken antibody clones in planta. Numbers (2-G5) refer to plants agroinfiltrated with the 10 different clones derived from combinatorial chicken phage libraries. YFP refers to plants agroinfiltrated with a non-specific insert (Yellow Fluorescent Protein). (A) ELISA to assess specific binding of chicken IgA in extracts of agroinfiltrated *N. benthamiana* leaves. IgA was bound to a crude mix of *E. acervulina* antigens and detected with an anti-α heavy chain antibody conjugated to peroxidase. Antibody binding is expressed as $OD_{403\,nm}$ with subtraction of background, which is measured by binding of an extract from leaves expressing a non-related protein. (B) Quantification of chicken IgA concentrations in crude extracts of agroinfiltrated *N. benthamiana* leaves by double sandwich ELISA. IgA was captured with anti-IgA antibody and detected with anti-light chain antibody conjugated to peroxidase to show assembly of heavy and light chains *in planta*. Antibody expression levels are expressed as percentage of total soluble protein (TSP) in the leaf extracts. In (A) and (B), bars represent average ± S.D. calculated from three independent plants. (C) Northern blot for analysis of transcription levels of plant immunoglobulin light and heavy chain mRNA. Ethidium bromide of ribosomal RNA (rRNA) shows sample loading. Lane "YFP" represents RNA from leaves infiltrated with a non-related construct (Yellow Fluorescent Protein).

10 clones assayed, the extracts from plants expressing the most abundant antibodies (numbers 10 and 11), also rendered the highest binding values. This was to be expected, since the binding activities of the original phage display clones from which the antibodies were derived all fell in the same range. However, binding activity did not always parallel expression levels (compare Fig. 2A and B) as can be seen for antibodies 2, 8 and G5. This can be a consequence of differences in binding capacities of some of the antibodies when expressed as Fab fragments in *Escherichia coli* compared to plant-produced full-size IgA antibodies.

As can be observed in Fig. 2B, drastic differences were found in the expression levels of the different antibody idiotypes. Indeed, most of them (6 out of 10) showed rather low expression levels. Northern analysis of agroinfiltrated leaves confirmed that antibody levels were not governed by the abundance of their respective mRNAs (Fig. 2C). Differential idiotypic expression has not been described in plants, yet the big range of yields reported for plant-made human and mice IgGs (generally derived from hybridoma technology) (Schillberg et al., 1999) could be an indication of the influence of variable regions on antibody stability *in planta*. This is also in agreement with data from other eukaryotic systems. Bentley et al. (1998), for instance, found up to 200-fold differences in expression levels of different antibody idiotypes when expressed transiently in CHO cells.

3.3. Chicken antibodies chains differ in their stability in planta

To gain insight in the differences in expression levels, recombinant IgA expression was followed by Western blot analysis under reducing conditions (Fig. 3A), using specific anti-chicken alpha heavy or light chain antibodies. As expected, control infiltrations with an expression cassette encoding a Yellow Fluorescent Protein (YFP) gave no signal, whereas chicken serum samples used as positive controls rendered two bands: a 75 kDa band corresponding to alpha heavy chain (IgHα) and a 23 kDa band which corresponded to chicken light chain (IgL). In contrast, plant-expressed IgAs resulted in a more complex pattern of bands. In most of the antibodies assayed, anti-IgHα antibody detected two major bands, labelled as H1 and H3 in Fig. 3. H1 size is compatible with a full length chicken IgHα, whereas H3 shows a MW of approximately 45 kDa and it is most likely the result of proteolytic degradation of H1. The presence of these smaller antibody fragments next to the full length form is a general

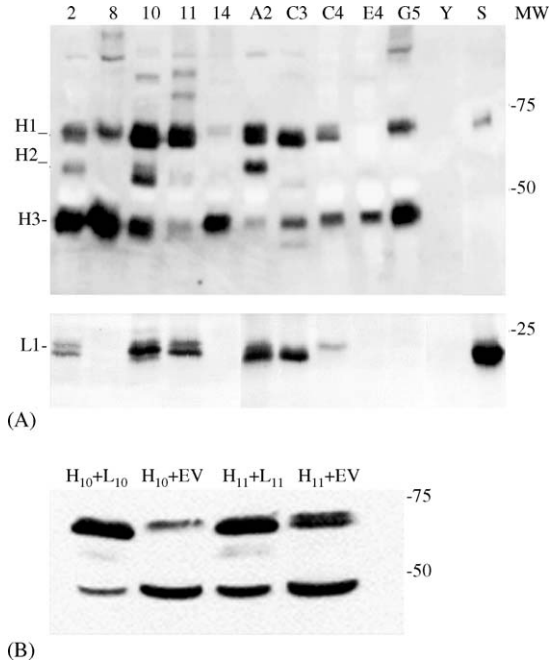

Fig. 3. Western analyses of plant-produced chicken antibodies. (A) Western blot analysis of 10 chicken antibodies expressed in *N. benthamiana* leaves (lanes 2–G5). Heavy chains and light chains were detected separately in the same crude extracts under reducing conditions. In lane S chicken serum was loaded as a positive control sample, Y lane represents protein extract from leaves infiltrated with a non-related construct (Yellow Fluorescent Protein). H1, H2 and H3 are labels for main heavy chain bands; L1 indicates bands detected by an anti-chicken light chain antibody. (B) Western analysis of two representative α heavy chain constructs co-expressed either with their partner light chain (lanes $H_{10}+L_{10}$ and $H_{11}+L_{11}$) or with a pBIN+-vector with no insert (lanes H_{10} + EV and H_{11} + EV). Molecular sizes are shown in kDa.

feature of plant-produced antibodies. A careful study by Sharp and Doran showed that murine IgG1 renders a complex pattern of fragments when expressed in plant cells, including fragments of heavy chains which apparently result from proteolytic degradation (Sharp and Doran, 2001). Occasionally, some clones (2, 10 and A2 in Fig. 3) showed an additional anti-IgHα reacting band (H2) located between H1 and H3, but the presence of this band was highly dependent on the extraction conditions and might represent incomplete dissociations between heavy and light chain fragments. Recombinant IgL expression was detected only in a limited number of cases (labelled as L1 in Fig. 3), namely clones 2, 10, 11, A2, C3 and C4.

We observed that those clones with H3 as the predominant α heavy chain band, also showed low or undetectable light chain expression. This was especially evident in clones 14 and E4 where H1 band was absent. This could indicate that the light chain stabilizes the α heavy chain in its full-size H1 form. To confirm this, we compared α heavy chain expression with or without partner light chain in two IgA constructs that produced well *in planta* (antibodies 10 and 11, respectively). As can be seen in Fig. 3B, the absence of a cognate light chain favored the accumulation of an H3 band in both examples. These observations suggest that the presence of an immunoglobulin light chain stabilizes the expression of full length α heavy chain and prevents its degradation into its H3 form. Our results suggest that poor expression of the light chain acts as a limiting factor in the stability of plant-made chicken IgA. Low IgL levels (as in clones 8, 14 and E4) correlate with IgHα fragmentation, low IgHα + IgL pairing as detected by sandwich ELISA and decreased binding activity. Reinforcing this view, we found in chain shuffling experiments that light chain substitution resulted in dramatic changes in antibody stability (data not shown).

It remains to be established which factors govern the observed differences in expression levels, particularly for the light chains. Since the use of transient expression discards transgene positional effects, we favor an explanation based on sequence diversity, which is concentrated in the CDRs of the variable regions. Among the antibodies analyzed in this study the variability is particularly high in CDR3 of both heavy and light chain, and comprises nucleotide substitutions as well as insertions and deletions (Wieland et al., 2006). We could not detect any feature that could explain yield differences in terms of codon usage. The most likely way in which variable regions are affecting immunoglobulin yield is by influencing protein sorting through the endomembrane system. In animal cells, antibody folding and assembly in the ER is—at least in part—determined by the sequence of the variable regions, probably mediated by their capacity to bind BiP and other chaperones (Skowronek et al., 1998). As a consequence, single amino-acid substitutions in VH or VL regions can result in impaired secretion and degradation (Wu et al., 1983; Dul and Argon, 1990; Chen et al., 1994; Martin et al., 1998). It is reasonable to think that some phage display-derived variable regions

whose performance has been selected in a prokaryotic system can present stability problems when confronted with the requirements of the plant endomebrane system.

Although general rules for plant-made chicken antibody stability can be pursued, in practical terms it is easier to choose empirically the best performer among several candidates. A "phage to plant" transfer system in combination with the agroinfiltration method allows testing dozens of antibody candidates within a relative short time.

3.4. Assembly of chicken dIgA and sIgA in planta

To test the ability of plant cells to assemble chicken secretory IgA complexes, expression cassettes containing chicken J-chain (JC) and secretory component (SC) were constructed. Chicken SC was cloned into a pBIN+-derived vector containing the murine kappa light chain secretion signal and 35S promoter. The SC clone comprises amino-acids 27–491 from chicken pIgR, therefore excluding its endogenous signal peptide and the transmembrane/cytosolic portion. Furthermore, a pBIN+ vector containing the chicken JC (with its endogenous signal peptide) was constructed. Plant expression of chicken antibody complexes was tested by co-infiltration of *N. benthamiana* leaves with combinations of *A. tumefaciens* cultures harboring chicken light chain (IgL), alpha heavy chain (IgH), with and without J-chain and/or secretory component as presented in detail in Fig. 4A. A Western blot analysis of leaf samples infiltrated with clone A2 is shown in Fig. 4B. Different high molecular weight bands were detected under non-reducing conditions. In all infiltrations, monomeric IgA with a size of approximately 180 kDa was detected, compatible with the size of serum-derived chicken IgA (Wieland et al., 2004). Co-infiltrations of IgA and J-chain resulted in the expression of a high molecular weight product with a molecular mass above 250 kDa, indicating formation of a dimeric IgA complex. Co-infiltrations of IgA and J-chain with secretory component caused a band with a reduced mobility compared to dIgA, demonstrating association between dIgA and the chicken SC. Co-infiltrations were assayed by mixing *Agrobacterium* cultures in different relative ratios (depicted in Fig. 4B). As shown in this figure, changes in the OD ratios did not substantially affect the formation of sIgA complexes. This is an indication of the high levels of co-transformation achieved with co-agroinfiltration and it evidences the robustness of the co-agroinfiltration technique for the assay of protein complex formation. No bands were observed when plants were infiltrated with YFP. Similar results were obtained when antibody IgAC3 was assayed, but not with IgA10 (data not shown). It is possible that the sequence of variable chains determines also their ability to assemble into the secretory complex. However, it seems more reasonable that sIgA formation is favored by a certain stoichiometry of the monomers, so that high expression

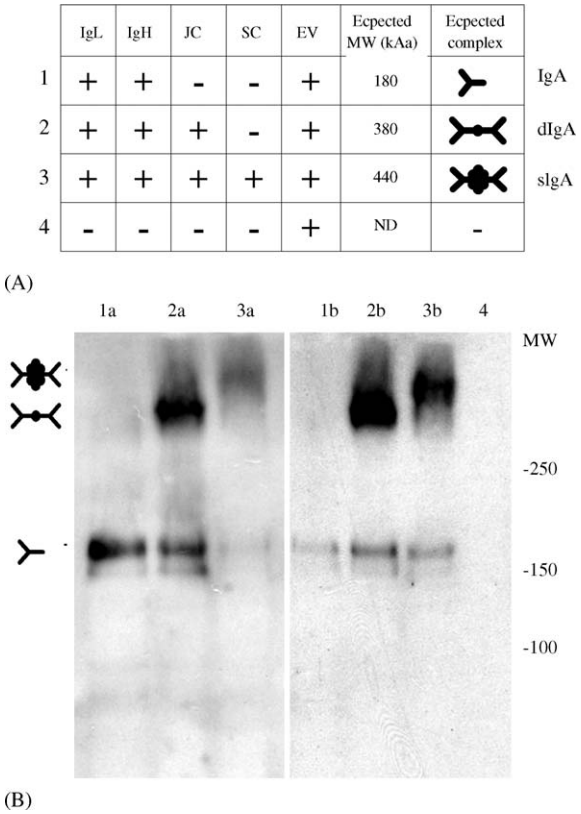

Fig. 4. Assembly of chicken dIgA and sIgA. (A) Table showing the agroinfiltration combinations used for plant-made sIgA assembly studies. (B) Western analysis of leaf samples agroinfiltrated as described in (A). In lanes labelled as "a", cultures were combined in a ratio (1:1:2:4) H + L + J + SC. In lanes labelled as "b" co-infiltration ratios were 1:1:1:1 (H + L + J + SC). In both cases the absence of a certain interaction partner was substituted by an equivalent amount of control culture (agrobacteria carrying empty pBIN+-vector). The molecular weight (on the left) is shown in kDa. The drawings give a schematic view of the detected IgA complexes.

levels of Ig chains complicate rather than facilitate the assembly of IgA into dIgA and sIgA. Further research is needed to determine the best conditions for chicken sIgA production in plants, including the requirements for accumulation in tissues other than leaves (e.g. seeds or fruits).

The formation of chicken sIgA complexes using plants as heterologous expression system serves as additional proof on the presence of sIgA in birds, which has been described only recently. The efficacy of plant-made sIgA in protective strategies has been previously demonstrated (Larrick et al., 2001). Extending molecular farming approaches to veterinarian medicine can accelerate the developments in the field by circumventing the difficulties associated with human clinical trials. In this regard, the demonstration that plants are able to assemble chicken sIgA opens the way for the use of animal feed plants as vectors for low-cost oral passive immunotherapy in chicken.

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