Plantibodies

Requirements for expression and subcellular targeting

Promotor: dr. ir. J. Bakker
hoogleraar in de Nematologie,
in het bijzonder in de fysiologie en moleculaire oecologie van nematoden

Co-promotoren: dr. ir. A. Schots
hoofd van het Laboratorium voor Monoklonale Antistoffen
dr. ir. F.J. Gommers
universitair hoofddocent, Departement Plantenveredeling en Gewasbescherming

513

Alexander Schouten

Plantibodies

Requirements for expression and subcellular targeting

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 30 oktober 1998
des namiddags te vier uur in de Aula.

The work presented in this thesis was performed at the Department of Nematology and the Laboratory of Monoclonal Antibodies (LMA) of the Wageningen Agricultural University, Binnenhaven 10, 6709 PD Wageningen, The Netherlands.

This research was financially supported by grants from the Netherlands Technology Foundation (STW), coordinated through the Foundation for Life Sciences (SLW). Additional support was obtained from EC grants BIO2-CT92-0239 and FAIR1-CT95-0905.

Schouten, A.

Plantibodies - Requirements for expression and subcellular targeting
Alexander Schouten
Thesis Wageningen Agricultural University – With references – With summaries in Dutch and English. Department of Nematology, P.O. Box 8123, 6700 ES The Netherlands.

ISBN 90-5485-916-4

Printed by: Ponsen & Looijen B.V., Wageningen

BIBLIOTHEEK
LANDBOUWUNIVERSITEIT
WAGENINGEN

Stellingen

1. De precisie en efficiëntie waarmee genen coderend voor immunoglobulinen, of domeinen hiervan, tot expressie gebracht kunnen worden in verschillende subcellulaire compartimenten van planten biedt perspectieven voor het creëren van nieuwe vormen van resistentie tegen tal van pathogenen en parasieten.

   Dit proefschrift

2. Het opmerkelijke feit dat 'single chain' antilichamen disulfidebruggen kunnen vormen in het cytosol van planten geeft aan dat de gangbare theorieën over de redoxpotentiaal van het cytosol toe zijn aan herziening.

   Thornton, J.M. (1981) *J. Mol. Biol.* 151, 261-287
   Gilbert, H.F. (1990) *Adv. Enzymol.* 63, 69-172
   Dit proefschrift

3. De 'short hinge' en 'long hinge' van de antilichamen van kamelen zijn uitermate geschikt voor de dimerisatie van 'single chain' antilichamen.

   Dit proefschrift

4. Gezien de functie van immunoglobulinen in het lichaam is de benaming 'antilichaam' nogal merkwaardig.

5. Door de insertie van ligand en receptor in het *genIII* van filamenteuze fagen kan de efficiëntie van 'phage display' voor het selecteren van eiwitvarianten aanzienlijk worden verbeterd.

6. Het grote aantal R-genen in aardappel met een verschillend werkingsspectrum doet vermoeden dat naast cellulasen diverse andere speekseleiwitten worden uitgescheiden door aardappelcystenaaltjes.

7. De uitspraak "Islands are often forcing houses of culture where small populations, their energies turned inwards, may originate experimental patterns of social organisation, reaching high levels of spiritual and artistic creativity." (*D. Carrington: Granite Island - A portrait of Corsica*, 1971) is ook van toepassing op de wetenschappelijke wereld.

8. Nematologen verschillen in die zin van nematoden dat persisters stressbestendig zijn.

9. Pseudo-macho's worden ontmaskerd in de behandelstoel van de tandarts.

   S.R. Uilenberg, persoonlijke mededeling

10. Vreemd genoeg is in de wetenschap het onverwachte vaak gewenst.

Stellingen behorende bij het proefschrift "Plantibodies - Requirements for expression and subcellular targeting" van Alexander Schouten

Wageningen, 30 oktober 1998

Abstract

Engineering resistance against pathogens is often frustrated by the lack of suitable genes. This problem can be overcome by *in planta* expression of antibodies (plantibodies) able to inactivate pathogen specific proteins. Since antibodies can be generated against almost any molecular structure, this strategy should be feasible for various diseases and pests.

Antibodies have several desirable features with regard to protein engineering. Using molecular techniques the relatively large antibody molecule (150 kDa), consisting of two heavy and two light chain molecules, can be trimmed to a single-chain antibody fragment (scFv) of 30 kDa, which consists of a variable heavy and light domain connected by a linker peptide.

This thesis describes the expression and subcellular targeting of functional full size antibodies and various types of scFv fragments in tobacco plants. The light and heavy chain coding genes of a full size model antibody were efficiently introduced in tobacco plants. A large number of the transformants expressed and secreted relatively high levels of the functional antibody.

From the model antibody an scFv fragment was derived and expressed in tobacco. The scFv was poorly secreted. However, the scFv fragments could be expressed intracellularly to high protein levels in the endoplasmic reticulum (ER) by adding the KDEL retention signal, and in the ectopic environment of the cytosol by omitting the ER translocation signal. Remarkably, the cytosolic scFv could only be expressed at detectable levels when the KDEL sequence was present. Immunoelectron microscopy and biochemical markers confirmed the proper subcellular locations of these intracellularly expressed scFv fragments. The KDEL extension also improved cytosolic expression levels of several other scFv fragments.

The *in vivo* redox state of these intracellularly expressed scFvs was determined. Both the variable heavy and light domains contain a disulfide bridge, which is considered crucial for stability and functionality of the scFv. As expected, these disulfide bridges were present in the oxidizing environment of the ER. Remarkably, in stable transformants the intramolecular disulfide bridges were also found in the scFvs present in the reducing environment of the plant cytosol, resulting in a functional antibody fragment. Like in the ER, the formation of intermolecular disulfide bridges was also observed, which resulted in functional dimers. When transiently expressed in the cytosol, the scFv was present in a reduced and non-functional form, confirming that the intramolecular disulfide bridges are necessary for the functionality.

In an attempt to improve scFv secretion levels, the model scFv was modified without changing the variable domains. The linker peptide was changed and modified, and expression levels were compared in transient expression assays. This only influenced the intracellular accumulation indicating that the secretory scFv was probably degraded somewhere in the secretory pathway. Various novel types of dimerized scFvs, by adding an extra domain derived from camel or IgM antibodies, were expressed transiently and in stable transformants. The addition of the camel IgG_2 'long hinge' in combination with the IgM C_H4 domain did significantly improve secretion.

It can be concluded that scFvs, unlike full size antibodies, can be targeted to various subcellular compartments. This allows direction of scFv fragments to locations where the pathogen specific protein is present. This thesis demonstrates that antibodies are versatile molecules and that engineering resistance through plantibody technology against nematodes, fungi and insects seems within reach.

'Voir plus loin que le bout de son nez.'

Voor Saskia, Thomas
en mijn ouders.

Contents

1 General Introduction 1

2 Coordinate expression of antibody subunit genes yields high levels of functional antibodies in roots of transgenic tobacco 9

3 The C-terminal KDEL sequence increases the expression level of a single chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco 23

4 Improving scFv antibody expression levels in the plant cytosol 39

5 Disulfide bridge formation within and between single-chain Fv antibodies in the reducing ectopic environment of the plant cytosol 53

6 ScFv stability in the secretory pathway of plant cells: Influence of linker peptides, the scFv architecture, C_H1 residues and the addition of camel hinges and IgM C_H4 domains 67

7 Summary and concluding remarks 95

References 103

Samenvatting 115

Dankwoord/ Acknowledgements 121

Curriculum vitae 123

CHAPTER 1

General Introduction

Advances in gene technology and plant transformation systems offer novel routes to engineer resistance. An appealing approach to obtain host plant resistance is to introduce genes encoding antibodies inhibiting specific factors (e.g. enzymes) essential for the pathogenesis. These factors can be present extracellularly, like cell wall degrading enzymes of fungi and nematodes (Smant *et al.*, 1998), or intracellularly, like viral proteins or factors involved in the induction of feeding sites by sedentary nematodes (*Globodera*, *Heterodera*, and *Meloidogyne* species). Consequently, antibody mediated resistance demands the expression of functional antibodies in the appropriate subcellular location. The aim of this thesis is to determine whether functional antibodies or antibody fragments can be efficiently expressed in various subcellular compartments of plants.

Function, origin and structure of antibodies.

Antibodies, or immunoglobulins, perform an essential role in the humoral immune response of vertebrates. Their unique function is to bind with high affinity to an apparently limitless variety of foreign invaders to neutralize and eliminate them from the body. Antibodies are synthesized by and initially displayed on the cell membrane of virgin B-lymphocytes. When triggered by an antibody inducing agent, or antigen, the organism reacts by B-lymphocyte proliferation resulting in antibody secreting plasma cells and memory cells, which reside in the spleen.

An antibody is a Y-shaped protein and is composed out of four subunits, two identical light chains and two identical heavy chains (Fig. 1A), both with a distinct domain structure. Each

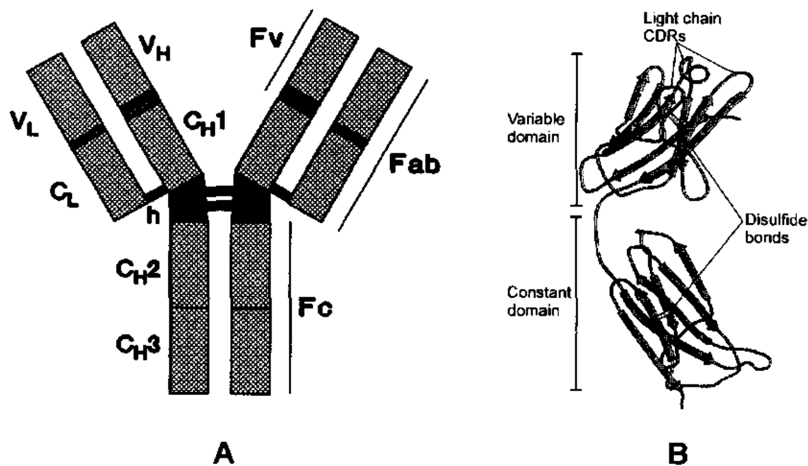

Fig. 1. A. Schematic representation of an IgG antibody molecule. The individual domains and the different fragments are indicated. Abbreviations used: C_H1- C_H3, heavy chain constant domains; C_L, light chain constant domain; V_L and V_H variable light and heavy chain, respectively; h, hinge region; Fv, variable fragment; Fab, antigen binding fragment; Fc, constant fragment. B. Ribbon model of the light chain variable and constant domains (Lodish *et al.*, 1995). The arrows represent the β–pleated sheet. The three complementarity-determining regions (CDRs) and intramolecular disulfide bridges are indicated.

chain contains a variable domain (V_L and V_H) at the N-terminal end of the peptide, which together form the variable fragment (Fv), followed by the constant domain(s), C_L of the light chain and C_H1, C_H2 and C_H3 of the heavy chain. Intermolecular disulfide bridges and non-covalent interactions hold the four subunits together as depicted in Fig. 1A. All domains are structurally similar and consist of three or four anti-parallel β strands, connected by loops of varying length (Fig. 1B). Three hypervariable loops, within each V_L and V_H domain mediate binding to a certain site (epitope) on the antigen. These complementarity-determining regions (CDRs) are formed during an ingenious recombination procedure during B cell maturation and determine the antibody specificity. Since each antibody contains two Fv fragments, it can bind two identical epitopes. For example, a mouse comprises initially about 10^8 different B-lymphocytes, each displaying an antibody with different specificity. This illustrates the vast number of different determinants that can be bound by antibodies. The constant domains serve different purposes. The C_H1 plays a crucial role during antibody assembly in the endoplasmic reticulum (ER). The interaction with the heavy-chain chaperoning protein (BIP) (Hendershot *et al.*, 1987) prevents the heavy chain from being secreted prior to pairing with the light chain. Next to holding the individual chains together by covalent and non-covalent interactions, the C_L and C_H1 domains extend the distance between the two antigen binding sites, facilitating cross-linking and agglutination (Dangl *et al.*, 1988; Schneider *et al.*, 1988). Antigen binding is further facilitated by a flexible hinge region, which increases the maximum rotation of the Fab arms (Fig. 1A). Since antibodies can bind with high specificity and affinity to an antigen they are potentially suitable for various scientific and medical applications. However, since the serum of an animal contains a wide variety of antibodies with different specificities, which can not be separated, the use of these polyclonal antibodies is limited.

Monoclonal antibodies.

The hybridoma technology (Köhler and Milstein, 1975) made it possible to immortalize individual antibody secreting B cells by fusing them to myeloma cells. The resulting hybrid myeloma cell, or hybridoma, has the characteristics of both cells: it secretes one type of antibody (monoclonal antibody) and grows indefinitely *in vitro*.

The hybridoma technology solves the problems encountered with polyclonal antibodies. Nowadays a wide variety of monoclonal antibodies are used as tools in diagnosis, therapy and research. A major drawback of the hybridoma technology is that it is only suited for the generation of murine antibodies, which are immunogenic in human therapy.

Inhibiting antibodies.

The high specificity and affinity of monoclonal antibodies has often also an another important impact on the antigen, which makes them even more valuable as tools for science and medicine. Some antibodies have the ability to inhibit the activity of the antigen upon binding (Fig. 2). This feature has been used to determine the topography of the active site of protein antigens. It has been well documented (Table 1) that monoclonal antibodies generated against an enzyme can inhibit the activity.

It was demonstrated by Carlson (1988) that antibodies can also be exploited to inhibit the *in vivo* activity of an antigen. Yeast (*Saccharomyces cervisiae*) cells expressing cDNAs encoding the heavy and light chain of an inhibitory anti-ADH (alcohol dehydrogenase) antibody displayed significantly reduced *in vivo* activity of this enzyme in the cytosol. This

result indicates that antibodies may also have the potential to provide resistance against pathogens or to alter metabolic pathways in plants.

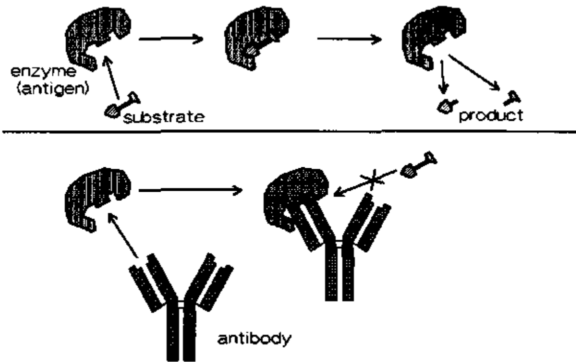

Fig. 2. Schematic representation of an inhibiting antibody. Binding of the antibody makes the active site of the enzyme inaccessible for the substrate and therefore prevents processing into a product.

Table 1. Examples of inhibition of enzyme activity by monoclonal antibodies (MAbs). For each of the protein antigens the number of raised MAbs and the number of inhibiting MAbs are shown.

Antigen	**Number of MAbs raised**	**Number of inhibiting MAbs**	**References**
Asparagine synthase	11	4	Pfeiffer *et al.*, 1987
Human pancreatic elastase 2	3	1	Shirasu *et al.*, 1988
Terminal deoxynucleotidyl transferase	4	3	Smith & Baumgarten., 1987
RNA polymerase II	5	3	Dahmus *et al.*, 1988
β-lactamase	9	5	Bibi & Laskov, 1990
Taq polymerase	9	8	Kellog *et al.*, 1994

Antibody engineering.

In medicine inhibiting antibodies can be used for therapy, like treatment against cancer. To reduce the immunogenicity of murine antibodies, 'humanization' through antibody engineering was necessary. This was facilitated by the domain structure of antibodies (Fig. 3). Initially, antibodies were humanized by replacing murine constant domains with those from human origin (Neuberger *et al.*, 1984 and 1985). These antibodies were still immunogenic. Human antibodies were then 'reshaped' by grafting the CDRs from a murine

on a human antibody (Jones *et al.*, 1986). Alternatively, to reduce antigenicity murine antibodies can be trimmed to functional $F(ab')_2$ and Fab fragments (Horwitz, *et al.*, 1988) (Fig. 3). Expression of only the V_H and V_L encoding genes in bacteria resulted in functional Fv fragments (Skerra and Plückthun, 1988). However, since the Fv fragment relies only on non-covalent interactions the stability is relatively low. This problem was overcome by constructing a single-chain Fv (scFv) gene in which the V_H and V_L domains were covalently coupled by a flexible linker peptide (Bird *et al.*, 1988; Huston *et al.*, 1988, Glockshuber *et al.*, 1990). In addition, this made cloning and expression less complicated since only one gene had to be expressed instead of two.

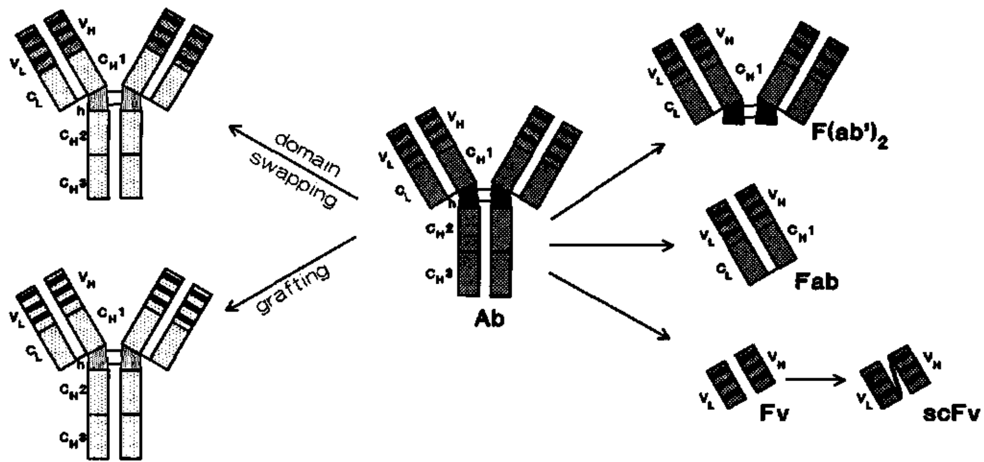

Fig. 3. The domain structure of antibodies facilitates engineering. Possibilities leading to a functional antibody fragment are indicated. For medical applications antibodies can be humanized by swapping the murine variable domains to human constant domains, or by grafting the complentairity-determining regions (CDRs; depicted as black boxes) on a human antibody. Fab and $F(ab')_2$ fragments can be obtained through proteolytic cleavage or gene technology. Fv and scFv fragments, in which the two variable Fv domains are connected by a peptide linker, can only be obtained through gene technology.

Antibody expression in heterologous systems.

Various heterologous expression systems for antibodies have been developed developed. Genes coding for full size antibodies and antibody fragments have been expressed, in yeast (Wood *et al.*, 1985; Horwitz *et al.*, 1988), myeloma cell lines (Orlandi *et al.*, 1989; Shin and Morisson, 1990), plants (Hiatt, *et al.*, 1989; Düring *et al.*, 1990), algae (Stieger *et al.*, 1991), insect cells (zu Putlitz *et al.*, 1990) and bacteria (Plückthun, 1991). The systems showed differences in expression level and the synthesis of functional antibodies.

Compared to eukaryotic expression systems bacterial expression is considered relatively simple and cheap to realize. However, in bacteria complete antibodies cannot be produced. Fab and scFv fragments can be expressed, although with limited success. The assembly into functional fragments proved to be the rate-limiting step in *E. coli*. Even when targeted to the periplasmic space, Fab and scFv fragments tend to aggregate in most cases as

incorrectly folded proteins, forming insoluble 'inclusion bodies'. Only low yields of properly folded and functional fragments are observed. From the inclusion bodies functional antibody fragments can only be recovered after time-consuming renaturation protocols.
In contrast, the eukaryotic expression systems do not show inclusion body formation. Although yields may be lower, these systems are capable of synthesizing full size antibodies and antibody fragments. The functional expression of these antibody genes may be caused by the presence of the proper molecular chaperones, which mediate the assembly of proteins, and prevent aggregation and misfolding of complete antibodies and antibody fragments.

Expression of antibodies in plants.
Expression of antibodies or antibody fragments in plants (plantibodies) can serve different purposes. Plants allow the production of antibodies on a large scale. It has been estimated that this production process is cheap when compared to expression in animal cells (Hiatt, 1990). The only concern is the potential cost of antibody purification from plants.
An interesting application is the use of antibodies to modulate traits of plants or to engineer resistance against pathogens. In this approach it is of paramount importance that the antibodies or antibody fragments can be expressed at sufficient levels at the proper subcellular location, i.e. the compartment where the antigen is present. So far, insufficient and confusing data were presented with respect to the expression levels of antibody and antibody fragments and the targeting of antibodies to different subcellular locations. Apparently, full size antibodies can not be expressed in the cytosol of plant cells (Hiatt *et al.*, 1989). The absence of suitable chaperones in the cytosol may prevent proper folding, leading to rapid protein degradation. Furthermore, the reducing environment may prevent intra- and intermolecular disulfide bridge formation, necessary for the assembly into stable and functional antibodies. IgG antibodies have been efficiently expressed in tobacco by targeting them into the secretory pathway using an immunoglobulin ER translocation signal (Hiatt, *et al.*,1989, Hein, *et al.*, 1991). However, Düring *et al.*, (1990) expressed low levels of an IgM antibody in tobacco, which was localized in the ER and chloroplasts when the α-amylase signal sequence for translocation into the ER was used. No secretion was observed. De Neve *et al.*, 1993 reported that expression of antibodies or Fab fragments differed significantly between transgenic tobacco and *Arabidopsis*, being very inefficient in the first and efficient in the latter (De Neve *et al.*, 1993). In addition only few plants showed high expression levels.
Data on the expression of scFv fragments are also scarce and confusing. ScFvs were efficiently expressed and secreted in transgenic tobacco plants (Firek *et al.*, 1993). Owen *et al.* (1992) had to analyze over one hundred transgenic tobacco plants to find one expressing an scFv fragment, which was expected to be located in the cytosol, at the relatively low level of 0.1%. In contrast, Tavladoraki *et al.* (1993) reported efficient expression of an scFv, targeted to the cytosol, which mediated resistance against artichoke mottled crinkle virus.
Altogether, this suggests that expression of plantibodies in plants is feasible, making them suitable molecules to obtain intracellular resistance and to modulate metabolic pathways.

Outline of this thesis.

This thesis presents results on the expression and subcellular targeting of complete antibodies and scFv fragments in plants. In chapter 2 an anti-cutinase antibody was efficiently expressed in tobacco plants by introducing one T-DNA carrying the coding genes of both the light and heavy chain, each containing the same ER translocation signal sequence and under control of a different promoter. As anticipated, the assembled antibodies were efficiently secreted.

In Chapter 3 tobacco transformants were obtained synthesizing scFv fragments, derived from the same anti-cutinase antibody, which were designed for secretion, retention in the ER, and expression in the cytosol. The four amino acid ER retention signal (KDEL) proves to retain the secretory protein in protoplasts. Surprisingly, this KDEL extension had also an effect on an scFv designed for expression in the cytosol. The KDEL sequence significantly improved the expression in the cytosol.

In Chapter 4 we confirm that the scFv antibodies targeted to the cytosol and ER are translocated to the expected subcellular locations. Furthermore, the beneficial effect of the KDEL retention signal was verified by adding this signal to other scFv fragments designed for expression in the cytosol.

In Chapter 5 the influence of the intra- and intermolecular disulfide bridges on the affinity for the antigen is determined for an scFv fragment present in the ER and the cytosol. The scFv fragment can form disulfide bridges in stable transformants and this is remarkable since the cytosol is considered a reducing environment in which disulfide bridge formation is supposed to be a rare event. These disulfide bridges prove to be crucial for scFv functionality.

In contrast to intracellular expression, low scFv protein levels were obtained in transgenic tobacco carrying the anti-cutinase scFv gene designed for secretion (see: Chapter 3). Chapter 6 describes the intracellular accumulation and degradation of scFv fragments during transit through the secretory pathway. Elements in the scFv fragments that may destabilize the protein, like the linker peptide and the C-terminus are modified in various ways to improve secretion levels. Also, it is determined whether the scFv secretion can be improved by adding an extra domain, responsible for multimerization. Addition of a camel hinge combined with an IgM C_H4 domain indeed improve secretion levels.

CHAPTER 2

Coordinate expression of antibody subunit genes yields high levels of functional antibodies in roots of transgenic tobacco.

This chapter was published in *Plant Molecular Biology* **26** (1994), 1701-1710, by the authors F.A. van Engelen, A. Schouten, J.W. Molthoff, J. Roosien, J. Salinas, W.G. Dirkse, A. Schots, J. Bakker, F.J. Gommers, M.A. Jongsma, D.Bosch and W.J. Stiekema.

Abstract

To explore the feasibility of employing antibodies to obtain disease resistance against plant root pathogens, we have studied the expression of genes encoding antibodies in roots of transgenic plants. A model monoclonal antibody was used that binds to a fungal cutinase. Heavy and light chain cDNAs were amplified by PCR, fused to a signal sequence for secretion and cloned behind CaMV 35S and TR2' promoters in a single T-DNA. The chimeric genes were cloned both in tandem and in a divergent orientation. The roots of tobacco plants transformed with these constructs produced antibodies that were able to bind antigen in an ELISA. Immunoblotting showed assembly to a full-size antibody. In addition, a $F(ab')_2$-like fragment was observed, which is probably formed by proteolytic processing. Both antibody species were properly targeted to the apoplast, but the full-size antibody was partially retained by the wall of suspension cells. The construct with divergent promoters showed a better performance than the construct with promoters in tandem. It directed the accumulation of functional antibodies to a maximum of 1.1% of total soluble protein, with half of the plants having levels higher than 0.35%. The high efficiency of this construct probably results from coordinated and balanced expression of light and heavy chain genes, as evidenced by RNA blot hybridization.

Introduction

Since its initial demonstration (Hiatt *et al.*,1989; Düring *et al.*, 1990), the expression of functional antibodies in transgenic plants has been considered highly promising for plant disease control and manipulation of metabolic pathways (Hiatt, 1990). This promise has been redeemed with reports of protection against virus infection (Tavladoraki *et al.*, 1993) and aberrant phytochrome-dependent germination (Owen *et al.*,1992) obtained through expression of antibody derivatives against viral coat protein or phytochrome, respectively.
Antibodies are heteromultimeric proteins composed of two heavy chains and two light chains connected by disulfide bridges. Thus, the production of functional antibodies requires the proper oligomerization of subunits in addition to gene expression. To promote antibody assembly and reduce the size of the molecule, antibodies have been engineered to form a single-chain Fv, in which the variable, antigen-binding domains of the light and the heavy chain are connected by a linker polypeptide (Bird and Walker, 1991). However, the affinity of the single-chain Fv for the antigen is often lower than the parent antibody (Bird and Walker, 1991; Tavladoraki *et al.*, 1993), which may be a serious drawback in applications requiring a high affinity.
In case of expression of complete antibodies, the synthesis of light and heavy chains should be balanced for antibody assembly. To obtain transgenic plants producing antibodies, light and heavy chain genes have initially been expressed in separate plants, which were subsequently crossed (Hiatt *et al.*, 1989). However, this procedure is time-consuming. Co-transformation is faster than crossing, but suffers from a large proportion of low antibody expressors (De Neve *et al.*, 1993). This may be due to independent variability of the expression of light and heavy chain genes. In addition, in both these approaches segregation will occur in the progeny if the two T-DNAs are not linked. Introduction of light and heavy chain genes on a single T-DNA circumvents the aforementioned disadvantages,

but puts additional demands on the promoter and terminator sequences employed. These sequences should preferably be different to avoid a potential lack of coordination of expression of the two transgenes (An, 1986; Dean *et al.*, 1988) or silencing of transgene expression in the progeny (Assaad *et al.*, 1993).
Because we aim for resistance against root pathogens, our research has been focussed on the expression of antibodies in plant roots, which have hardly been studied for antibody expression so far. On the basis of the above considerations, we have chosen to transfer the antibody genes on a single T-DNA under the control of the cauliflower mosaic virus 35S promoter (heavy chain) and the TR2' promoter (light chain), which both have a high activity in roots (Benfey *et al.*, 1989; Langridge *et al.*, 1989; Teeri *et al.*, 1989). As a model, we have employed the monoclonal antibody 21C5 that was raised against a cutinase produced by the fungus *Botrytis cinerea* (Salinas, 1992). The analysis reported here shows coordinate and balanced expression of light and heavy chain genes giving rise to a large proportion of tobacco plants with a high level of antibodies (up to 1.1% of total soluble protein) in the roots. These antibodies are targeted to the apoplast and bind to the target enzyme. Thus, we have created an efficient system for high-level expression of antibodies in roots that has the potential to interfere with pathogenesis.

Materials and methods

cDNA amplification and vector construction
Messenger-RNA was isolated from the 21C5 hybridoma (Salinas, 1992) by oligo(dT)cellulose chromatography using the QuickPrep *Micro* mRNA Purification Kit from Pharmacia. First strand cDNA was synthesized using the First Strand cDNA Synthesis Kit from Pharmacia. The antibody cDNAs were amplified from this material using the following primers: light chain: 5'-GGTGTCGACGGTGATGTTGTGATGACCCAATCTCC-3'(VL) and 5'-CGT-CAGATCTTTAACACTCATTCCTGTTGAAGCTC-3'(CL); heavy chain: 5'-GGTGTCGACTGTGAGG-TCCAGCTGCAACAATCTG-3' (VH) and 5'-GAGGTTAACTTATTTACCAGGAGAGTGGGAGAGGCT-CTT-3' (CH3). 0.5 μl of the first strand cDNA synthesis reaction was amplified with 12.5 pmol of each primer in 20 mM Tris pH 8.8, 10 mM KCL, 6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.1% Triton X-100, in a total volume of 50 μl. After 5 min denaturation at 94°C and 3 min annealing at 50°C 1.25 units *Pfu* DNA polymerase (Stratagene) was added, followed by 34 cycles consisting of 3 min 72°C, 1 min 94°C and 2 min 50°C. A polymerisation step of 5 min 72°C concluded the amplification. PCR products of the correct size were excised from agarose gels and purified by adsorption to Prep-a-Gene (Bio-Rad) glass beads. The fragment ends were made blunt with Klenow DNA polymerase (Sambrook *et al.*, 1989) and cloned into the SmaI site of pBluescript SK+ (Stratagene). After sequence analysis (see below) the inserts were cut from the plasmids with *SalI*/*HpaI* (heavy chain) or *SalI*/*Bam*HI (light chain). An DNA fragment encoding a signal peptide for secretion was constructed by annealing and ligation of four oligonucleotides. This sequence was derived form the signal sequence of the kappa light chain of antibody CEA 66-E3 (Kabat *et al.*, 1991) by changing the codons at positions -3 and -2 from GTTGAA to GTCGAC, which converted the glutamic acid residue at -2 to aspartic acid. This NcoI/SalI fragment was ligated to the heavy chain and cloned between the CaMV 35S promoter with a duplicated enhancer (Kay *et al.*, 1987) and the nopaline synthase (NOS) terminator of the binary vector pCPO31. This vector is identical to the previously described pCPO5 vector (Florack *et al.*, 1994) except for an

additional *Nco*I site downstream of the 35S promoter. To introduce the light chain cDNA into this vector, a *Hin*dIII fragment containing the TR 2'-1' dual promoter and 35S terminator was first cloned into the *Hin*dIII site of pBluescript (Stratagene) from which the BamHI site had been deleted. This plasmid was cut between the TR2' promoter and 35S terminator with *Nco*I and *Bam*HI and ligated to the light chain fragment and signal sequence. Then the *Hin*dIII fragment was transferred to pCPO31.
DNA sequences were determined on an Applied Biosystems 370A automated DNA sequencer by PCR cycle dideoxy chain termination sequencing using fluorescently labeled dideoxynucleotides. The obtained sequences were analyzed with the gcg DNA sequence analysis software package (Devereux *et al.*, 1984). The nucleotide sequence data were deposited in the GSDB, EMBL, Genbank and DDBJ databases under the accession numbers L35037 and L35138.

Plant transformation
Constructs were first transferred to *E. coli* strain S17.1 and subsequently to *Agrobacterium tumefaciens* strain GV3101 (pMP90RK) (Koncz and Schell, 1986) by biparental mating. Tobacco (*Nicotiana tabacum* cv. Samsun NN) leaf discs were transformed essentially according to the method of Horsch *et al.* (Horsch *et al.*, 1985). Minor modifications included incubation of the leaf discs with *Agrobacterium* culture for three days in liquid medium containing 1 mg/l NAA and 0.2 mg/l BAP and the use of cefotaxime (Duchefa, Haarlem, The Netherlands) to control bacterial growth after transformation. Stably transformed plants were maintained under sterile conditions on MS agar medium (Duchefa) containing 3% (w/v) sucrose and subsequently transferred to soil in the greenhouse. For protein and RNA analyses, plants were dissected, frozen in liquid nitrogen and stored at -80°C. Roots taken from agar were rinsed with water to remove most of the attached agar prior to freezing.

Protein extraction and analysis
Plant tissues were ground with mortar and pestle in liquid nitrogen. The fine powder was added to an equal volume of 2xPBS (phosphate-buffered saline) and thawed. After mixing, samples were centrifuged twice to remove insoluble material. Protein levels were determined with the Coomassie Brilliant Blue assay from Bio-Rad, using bovine serum albumin (fraction V, Sigma) as standard. For immunoblot analysis proteins were separated by standard SDS-PAGE and blotted onto nitrocellulose membranes (Sambrook *et al.*, 1989). These were blocked with PBS containing 4% (w/v) nonfat dry milk and 0.2% (v/v) tween-20, incubated with sheep-anti-mouse IgG or rat-anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories) conjugated to alkaline phosphatase and developed with NBT and BCIP as substrates (Sambrook *et al.*, 1989).
To assay for antigen binding, microtiter plates were coated overnight with 250 ng per well of a partially purified cutinase preparation in 50 mM sodium carbonate, pH 9.6 at 4°C. Wells were washed twice with PBS, blocked for four hours at room temperature with 5% (w/v) nonfat dry milk in PBS and washed twice with PBS containing 1% (w/v) nonfat dry milk (PBSM). Either one to five μg of plant proteins or a known concentration range of 21C5 standard monoclonal antibody, both in PBSM, were added to the wells and incubated overnight. The plates were washed four times with PBSM. To determine the antigen binding capacity of full-size and processed antibodies qualitatively the wells were subsequently washed with PBS, eluted with SDS-PAGE sample buffer and analyzed by immunoblotting. Alternatively, for quantitative ELISA, wells were incubated for two hours with alkaline

phosphatase-conjugated sheep-anti-mouse IgG antibodies (F(ab')$_2$-specific, Jackson ImmunoResearch Laboratories). After four washes with PBSM and two with PBS, the plates were developed with 0.75 mg/ml 4-nitrophenylphosphate in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$.

Protoplast and cell suspension culture methods

Prior to plant transformation, constructs were tested by transient expression in tobacco (*Nicotiana tabacum* cv. Samsun NN) leaf protoplasts using polyethylene glycol as described by Denecke *et al.* (1989). The same protoplast isolation and culture method was employed to study secretion. Proteins secreted into the culture medium in these experiments were precipitated with ethanol. Protein pellets and protoplasts were dissolved in SDS-PAGE sample buffer. A cell suspension culture was initiated from transgenic stem slices. Callus formation was induced on MS agar plates containing 3% sucrose, 2 mg/l IAA, 0.5 mg/l kinetin and 2 g/l casein hydrolysate. Friable callus was transferred to liquid MS medium containing 2 mg/l 2,4-D, 0.1 mg/l kinetin and 1 g/l casein hydrolysate and subcultured every 10 to 14 days. Cells were separated from culture medium by sieving, washed with fresh culture medium and ground in protein sample buffer. Culture medium was filtered through a 0.22 μm filter and treated as described above for protoplast culture medium.

RNA and DNA isolation, blotting and hybridization

RNA was isolated as described by De Vries *et al.* (1991). Five μg of each RNA preparation was denatured with DMSO and glyoxal (Sambrook *et al.*, 1989), separated on a 1% agarose gel, blotted onto Gene Screen Plus membrane (New England Nuclear) and hybridized to ^{32}P-labeled DNA probes in formamide-containing buffer at 42°C according to the manufacturer's instructions. The stringency of the final washing was 2xSSC at 65°C. The blot was successively hybridized with the complete light chain cDNA, with a subclone of the heavy chain cDNA comprising the variable domain and the first constant domain and with an rDNA probe. Probes were labeled with [α-^{32}P]-dATP by random primed labeling (U.S. Biochemical corp.).

Results

Cloning of 21C5 antibody cDNA and transformation of tobacco

The 21C5 monoclonal antibody employed in this study is an IgG1, kappa isotype antibody that binds to a cutinase produced by *Botrytis cinerea* (Salinas, 1992). The cDNA sequences encoding the heavy and light chain of 21C5 were amplified using cDNA prepared from total 21C5 hybridoma mRNA. To increase the fidelity of 5'-end amplification, eight different primers based on the 5'-end of variable domain sequences of both heavy and light chains, as found in the Kabat database (Kabat *et al.*, 1991), were tried. The PCR reaction with the highest yield was considered to contain the best matching primer set and the product was used for cloning and sequence analysis. Comparison of the sequences obtained with sequences in an immunoglobulin database (Kabat *et al.*, 1991) confirmed that the heavy chain was an IgG1 and the light chain was of the kappa type. The 21C5 cDNAs were fused to a fragment encoding a slightly modified antibody signal sequence (Fig. 1A; Kabat *et al.*, 1991) and cloned into the plant expression vector pCPO31 (Fig. 1B). In the resulting construct a 35S promoter with duplicated enhancer (Kay *et al.*, 1987) drives the expression

A

NcoI ... SalI

CCATGGGCATCAAGATGGAGACACATTCTCAGGTCTTTGTATACATGTTGCTGTGGTTGTCTGGTGTCGAC

M G I K M E T H S Q V F V Y M L L W L S G V D

B

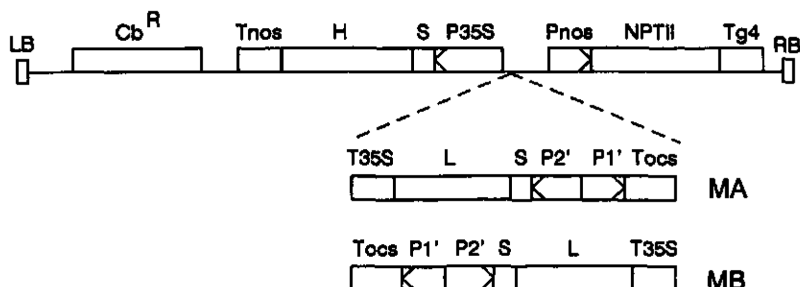

Fig 1. A. Secretory signal sequence cloned in front of heavy and light chain cDNA. Restriction enzyme cleavage sites are shown above the sequence and the amino acid translation below the sequence.
B. T-DNA constructs. LB, left border; RB, right border; L, light chain gene; H, heavy chain gene; S, secretory signal sequence; NPTII, neomycin phosphotransferase gene; Pnos, nopaline synthase promoter; Tg4, T-DNA gene 4 terminator; P2', T-DNA gene 2 promoter; T35S, CaMV 35S terminator; P35S, CaMV 35S promoter with doubled enhancer; Tnos, nopaline synthase terminator; Cb^R, carbenicillin resistance gene. The T-DNA gene 1 promoter (P1') and the octopine synthase terminator (Tocs) were not used in this study.

of the heavy chain (type "M" construct), while the light chain expression is under the control of the TR2' promoter. The cloning procedure generated two different orientations of the TR2'-light chain cassette with respect to the 35S-heavy chain cassette (Fig. 1B). In the MA construct the distance between the transcription initiation sites of the 35S and the TR2' promoter was 1730 bp and the genes were transcribed in tandem. In the MB construct this distance was 1340 bp and transcription was divergent. ELISA analysis of transient expression products from tobacco protoplasts confirmed that the heavy and light chains encoded by these constructs could form functional antibodies (data not shown). Both constructs were transferred to tobacco by *Agrobacterium tumefaciens*-mediated leaf-disc transformation.

21C5 antibodies are functional and partly processed in tobacco roots

The production of antibodies in roots of transgenic tobacco plants was analyzed by immunoblotting of root extracts from a randomly selected set of axenically grown plants. Under non-reducing conditions, a protein of the size of a fully assembled antibody could be specifically detected in most samples with an anti-mouse-IgG secondary antibody (Fig. 2A). In addition, another prominent protein band with an estimated size of about 110kDa and two other, faint bands were visible on the blot. Upon reduction with DTT none of these bands could be detected (results not shown), showing intermolecular disulfide bonds to be present in these proteins.

To identify the nature of the 110 kDa protein, immunoblotting was performed with secondary antibodies that recognize different domains of immunoglobulins (Fig. 2B). Whereas the band running at the position of a full-size antibody reacted with antisera specific for either light

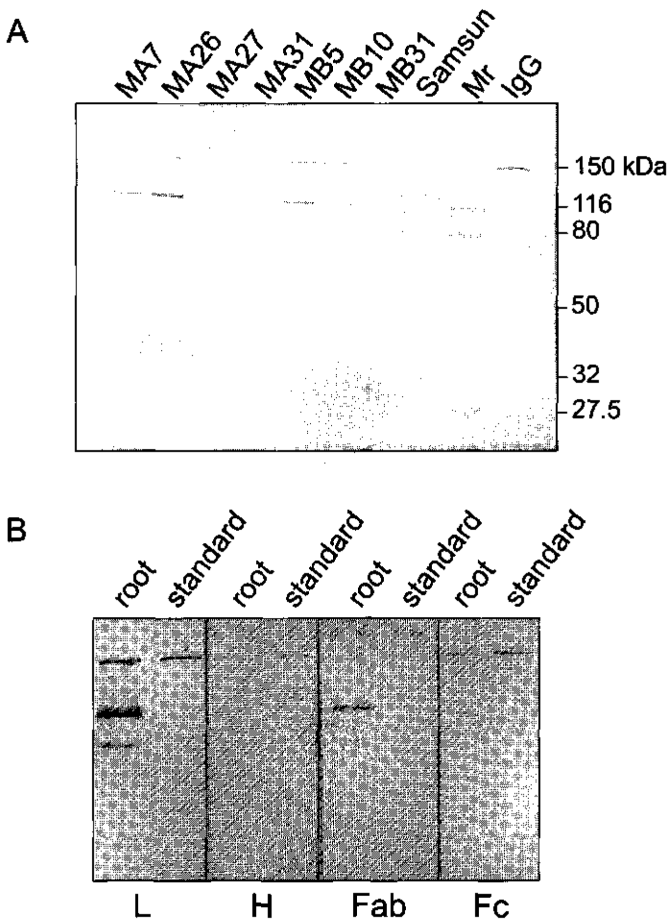

Fig 2. A. Immunoblot of root extracts from different MA and MB plants, developed with an anti-mouse IgG secondary antibody. The lane marked "Samsun" is from an untransformed control plant. "IgG" is an immunoglobulin G standard purified from hybridoma culture supernatant. "Mr" is a prestained molecular weight marker set. B. Immunoreactivity of antibodies in a root extract (lanes marked "root") or a standard hybridoma IgG (lanes marked "standard") with secondary antisera specific for light chain (L), heavy chain (H), Fab fragment (Fab) or Fc fragment (Fc).

chain, heavy chain, Fab or Fc fragments, the 110 kDa protein could be detected with all antisera except for the anti-Fc reagent. Thus, it is most likely that the 110 kDa protein has a structure similar to an $F(ab')_2$ fragment, a dimer of two Fab fragments held together by disulfide bridges.

To determine if both these forms of plant-produced antibodies were functional, the binding of the antibodies to immobilized cutinase was assayed (Fig. 3). This revealed that both the full-size and the 110 kDa antibody specifically bind to the cutinase antigen, which further supports the $F(ab')_2$-like structure of the 110 kDa fragment.

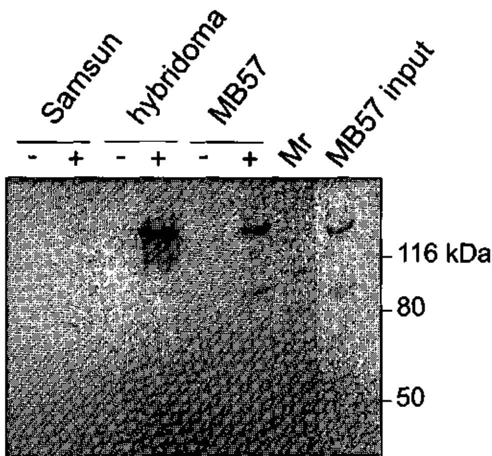

Fig 3. Binding of antibodies from a transgenic plant to cutinase. Immunoblot of proteins eluted from wells coated with (+) or without (-) cutinase after incubation with root extract from untransformed control plant ("Samsun"), 21C5 antibody from hybridoma culture supernatant ("hybridoma") or root extract from plant transformed with MB construct ("MB57"). The right lane shows the antibodies present in the MB57 extract before addition to the wells.

Secretion of antibodies

To study secretion of antibodies to the apoplast, we isolated protoplasts from leaves of an MB plant with a high antibody expression. The protoplasts were cultured in fresh medium for 0.2 to 20 hours and the culture medium and cells were analyzed by immunoblotting (Fig. 4, left panel). Antibodies accumulated in the culture medium with time, while in the cells, of which more than 90% remained viable, antibodies were not detectable at any time point. Thus, the antibodies are secreted by the plant cells. Furthermore, mainly full-size (150 kDa) antibodies were detected, suggesting that the formation of the 110 kDa $F(ab')_2$-like fragment *in planta* occurs only after secretion and is most likely due to proteolytic processing in the apoplast.

The fate of the antibodies after secretion was investigated in a cell suspension culture of the same plant (Fig. 4, right panel). Only the 150 kDa full-size antibody was associated with the cells in this culture, whereas both the 150 kDa antibody and the $F(ab')_2$-like fragment were observed in the culture medium. This suggests that the 110 kDa $F(ab')_2$-like fragment can freely diffuse through the cell walls of suspension cells, while the full-size antibody is partially retained by these walls.

Promoter configuration affects expression level

To compare the efficacy of the two constructs used, the level of functional antibodies was determined in root extracts by ELISA. Since different root extracts contained full-size and $F(ab')_2$-like antibody molecules in different ratios (Fig. 2a), expression levels could only be properly quantified with Fab-specific secondary antibodies. The data obtained are summarized in Figure 5 and Table 1. They show functional antibody expression for both constructs, the highest levels being 0.6% and 1.1% of total soluble protein for the MA and MB constructs, respectively. The between-transformant variability of expression levels was large, a phenomenon commonly observed with primary transgenic plants. Because the MB

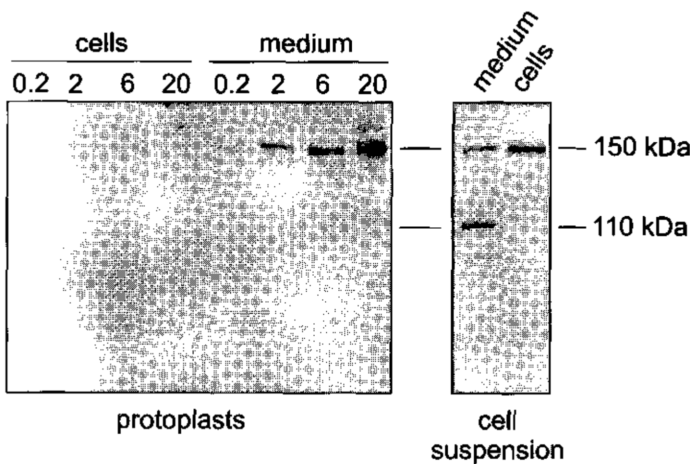

Fig 4. Secretion of antibodies by plant cells. Left panel. Immunoblot detection of antibodies in total cellular or culture medium proteins from a transgenic protoplast culture grown for 0.2, 2, 6 or 20 hours, as indicated on top of the lanes. Right panel. Immunoblot detection of antibodies in total cellular or culture medium from a transgenic cell suspension culture grown for four days.

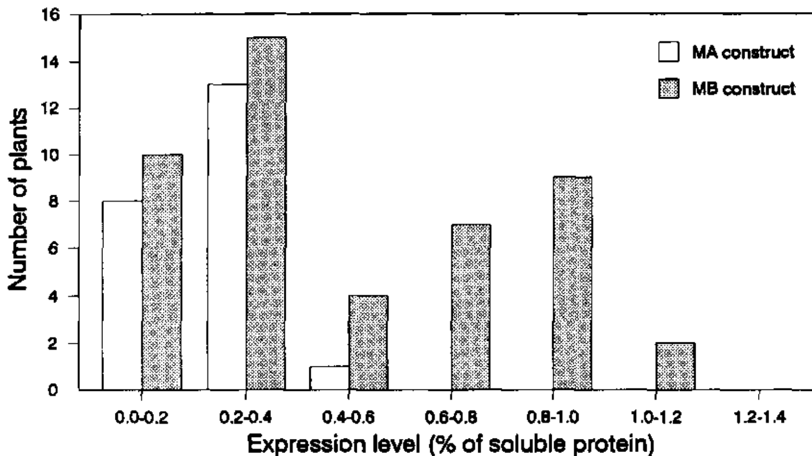

Fig 5. Frequency distribution of antibody expression level in roots of MA and MB primary transgenic plants. The expression level is given as percentage of total soluble root protein.

population was not normally distributed, the non-parametric Wilcoxon-Mann-Whitney test was used to evaluate the differences between the populations (Neave and Worthington, 1988). This demonstrated that the MB construct gave significantly higher expression levels than the MA construct. Comparison of the mean values suggested that the difference was 2-fold. However, in the case of non-normality the significance of means is uncertain and median values are more informative (Nap *et al.*, 1993). The medians indicated a 1.5-fold

higher expression for the MB construct. The difference between the two constructs was further underlined by the fact that 35% of the MB plants showed an expression level above 0.6%, while this figure was 0% for the MA plants.
Stems, leaves and flowers from plants with a high antibody expression in roots were also found to contain antibodies, with levels ranging from 0.05% to 0.4% (data not shown).

Table 1. Expression level statistics.

	MA	MB
number of plants	22	50
mean	0.24%	0.45%
highest	0.58%	1.1%
median	0.24%*	0.36%*

* significantly different ($p < 0.0006$) according to Wilcoxon-Mann-Whitney test

Coordinated and balanced expression of heavy and light chain genes

The expression levels of two genes on a single T-DNA have been found to exhibit independent between-transformant variability in a number of cases (An, 1986; Jones *et al.*, 1987; Dean *et al.*, 1988; Peach and Velten, 1991). In addition, the use of different promoters may lead to unequal expression of these two genes. In the case of antibodies, unbalanced expression would be highly undesirable, since functional antibodies will only be produced to high levels when expression of *both* heavy and light chain genes is high. To evaluate the behaviour of our expression system in this respect, we determined the relative expression levels of light and heavy chain genes in roots by RNA blot hybridization. Plants from three different expression categories, low (0.005%-0.01%), medium (0.05%-0.1%) and high (0.5%-

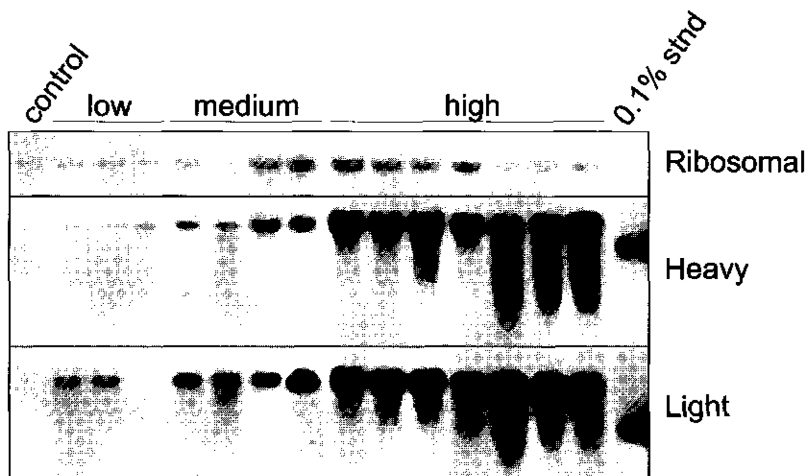

Fig 6. RNA gel blots containing total RNA from plants with a low, medium or high level of antibodies (see text) hybridized with probes specific for ribosomal RNA (upper panel), heavy chain (middle panel) or light chain (lower panel) sequences. In the rightmost lane an amount of DNA encoding the mature heavy or light chain was applied corresponding to 0.1% of mRNA. In the leftmost lane RNA was applied from a plant transformed with an empty vector.

1%), were chosen for this analysis. This experiment showed that the expression of light and heavy chain genes covaries between different independent transformants (Fig. 6). Comparison of the signal intensities of the root mRNA bands with the intensity of hybridization to a known amount of light chain or heavy chain cDNA revealed that the absolute heavy and light chain mRNA levels in individual transformants were similar.
Furthermore, a correlation was observed between the amount of mRNA and the level of assembled antibody as determined by ELISA. Overall, the construct employed directs both coordinate and balanced expression of antibody subunit genes, explaining the large proportion of plants accumulating a high level of assembled, functional antibody in roots (Table 1).

Discussion

Antibody-mediated inactivation of antigens provides new possibilities for resistance breeding in plants. This was recently demonstrated by the inhibition of AMCV infection by expression of an scFv antibody in the leaves of *Nicotiana benthamiana* (Teeri *et al.*, 1989). In this paper we describe an efficient transformation vector that directs high level expression of functional antibodies in roots of transgenic tobacco. Controlled by the CaMV 35S and TR2'promoters on a single T-DNA, expression of the heavy and light chain genes is coordinated and balanced with this construct, giving rise to expression levels exceeding 0.35% of total soluble root protein in more than half of the transgenic plants obtained. Obviously, this expression system could be employed for the simultaneous high level expression of other proteins in roots of transgenic plants.
Transgene expression level variability between independent transformants is commonly observed in plant transformation experiments. When two transgenes have to be expressed simultaneously, independent variability of these genes may be limited by transferring them on one T-DNA. However, from a number of studies it has become clear that even this approach does not warrant concurrent expression of the two genes (An, 1986; Jones *et al.*, 1987; Dean *et al.*, 1988; Gidoni *et al.*, 1988; Peach and Velten, 1991). In one study the expression from two different and two identical promoters was directly compared in the same T-DNA background (Dean *et al.*, 1988). In this case coordinated expression was only observed when different promoters were used. We used the 35S and TR2' promoters to direct the expression of the heavy and light chain, respectively, and observed covariation of light and heavy chain mRNA levels, which is in line with those observations. In other studies, however, the use of different promoters was not sufficient to achieve coordinated expression (Jones *et al.*, 1987; Peach and Velten, 1991).
In addition to covariation, the absolute expression levels of light and heavy chain genes must be similar and the subunits must be produced in the same cell types to achieve a high level of assembled antibody. The combination of 35S and TR2' promoters seemed particularly useful in this respect, since these promoters show a similar tissue-specific pattern and level of reporter gene expression in roots (Benfey *et al.*, 1989; Langridge *et al.*, 1989; Leung *et al.*, 1991; Teeri *et al.*, 1989). The high antibody expression we observe in roots demonstrates that the spatial expression patterns of these promoters indeed coincide.
We have shown that construct MB with divergent promoters has a better performance than construct MA with promoters in tandem. It could be argued that this difference is due to different susceptibility of the two constructs to influences of neighbouring plant DNA (Breyne

et al., 1992). However, a similar difference was observed in transient expression assays in protoplasts (data not shown), which do not involve integration in the plant genome and, therefore, cannot be susceptible to position effects. Therefore, we consider it more likely that the effect is caused by the enhancer action that has been described for the doubled 35S promoter (Kay *et al.*, 1987). This enhancer activity is influenced by distance, orientation and up- or downstream location (Odell *et al.*, 1988). In the MB construct the 35S enhancer is 400 base pairs closer to the TR2' promoter, located upstream (downstream in MA) in a divergent orientation (tandem in MA). This configuration could stimulate transcription from the TR2' promoter more than the A-configuration, thus significantly increasing the amount of antibodies produced.

The proper targeting of antibodies will be crucial for their effectiveness against pathogens. Therefore, we studied the secretion of antibodies in transgenic plants. Murine secretory signal sequences appear to be well-suited to direct antibodies to the apoplast of plant cells. This was previously shown (Hein *et al.*, 1991) and is confirmed here by the accumulation of fully assembled immunoglobulins in protoplast culture medium. Once in the apoplast, antibodies appear to be subject to proteolytic processing, as indicated by the occurrence of a 110 kDa $F(ab')_2$-like antibody fragment in a cell suspension and in root tissue. Similar results were obtained by other authors (Hiatt *et al.*, 1989; De Neve *et al.*, 1993), but not further investigated. If the formation of this fragment is indeed due to proteolytic cleavage, it is not surprising that cleavage occurs in the so-called "hinge" region between the Fab and Fc domains, since this is a highly flexible and accessible domain that is cleaved by the proteases pepsin and papain as well (Amzel and Poljak, 1979). The formation of 110 kDa antibody fragments apparently influences the fate of antibodies after secretion: full-size antibodies are somewhat retained by the walls of suspension cells, but the $F(ab')_2$-like fragments can freely diffuse. However, since the cell wall porosity of suspension cells and cells *in planta* may differ, care must be taken in extrapolating these results to cells in intact plant tissues.

Although antibodies are susceptible to proteolytic processing, the products are functional and stable in plant roots, as suggested by the high level of functional antibody accumulation. This provides a basis for the application of antibody technology in resistance breeding.

Acknowledgements

We thank Jacoline Zilverentant and Marga van Gent-Pelzer for technical assistance, Jan-Peter Nap for helpful discussions and assistance in computer analysis of sequence data, and Dick Geurtsen for plant care in the greenhouse. This work was financially supported by a grant from the Netherlands Technology Foundation (STW), and was coordinated by the Foundation for Life Sciences (SLW).

CHAPTER 3

The C-terminal KDEL sequence increases the expression level of a single chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco

This chapter was published in *Plant Molecular Biology* **30** (1996), 781-793, by the authors A. Schouten, J. Roosien, F.A. van Engelen, G.A.M. de Jong, A.W.M. Borst-Vrenssen, J.F. Zilverentant, D. Bosch, W.J. Stiekema, F.J. Gommers, A. Schots and J. Bakker.

Abstract

The effects of subcellular localization on single chain antibody (scFv) expression levels in transgenic tobacco was evaluated using an scFv construct of a model antibody possessing different targeting signals. For translocation into the secretory pathway a secretory signal sequence preceded the scFv gene (scFv-S). For cytosolic expression the scFv antibody gene lacked such a signal sequence (scFv-C). Also, both constructs were provided with the endoplasmic reticulum (ER) retention signal KDEL (scFv-SK and scFv-CK, respectively). The expression of the different scFv constructs in transgenic tobacco plants was controlled by a CaMV 35S promoter with double enhancer. The scFv-S and ScFv-SK antibody genes reached expression levels of 0.01% and 1% of the total soluble protein, respectively. Surprisingly, scFv-CK transformants showed considerable expression of up to 0.2% whereas scFv-C transformants did not show any accumulation of the scFv antibody. The differences in protein expression levels could not be explained by the steady-state levels of the mRNAs.
Transient expression assays with leaf protoplasts confirmed these expression levels observed in transgenic plants, although the expression level of the scFv-S construct was higher. Furthermore, these assays showed that both the secretory signal and the ER retention signal were recognized in the plant cells. The scFv-CK protein was located intracellularly, presumably in the cytosol. The increase in scFv protein stability in the presence of the KDEL retention signal is discussed.

Introduction

Recent advances in antibody engineering offer various perspectives to endow plants with new properties. Antibodies and antibody fragments can be used to engineer disease resistance, to alter or design metabolic routes with catalytic antibodies, and to study plant growth and development by antisense-like approaches (Schots *et al.*, 1992). For these applications it is crucial to have functional antibodies located in the proper subcellular compartment. This can be accomplished by providing the antibody with suitable targeting and sorting signals (Biocca *et al.*, 1990).
The engineering of antibodies is facilitated by their domain structure. The domains carrying the antigen-binding loops can be manipulated in different ways to create various biologically active fragments (Winter and Milstein, 1991). An interesting and valuable antibody fragment is the single chain antibody (scFv), in which the variable domains of light and heavy chain are connected by a flexible peptide linker. Through expression of scFvs, several problems inherent to the post translational processing of full size antibodies, like assembly of the four subunits, formation of intermolecular disulfide bonds, and glycosylation, can be circumvented (Hiatt *et al.*, 1989; Hein *et al.*, 1991).
Single chain antibodies have been successfully expressed in plants. Constitutive cytosolic expression of an scFv antibody in tobacco mediated resistance against artichoke mottled crinkle virus (Tavladoraki *et al.*, 1993). Owen *et al.* (1992) and Firek *et al.* (1993) reported cytosolic expression and secretion of an anti-phytochrome scFv antibody.
Cytosolic expression of functional scFv antibodies in plants and other eukaryotes (Biocca *et al.*, 1993 & 1994; Werge *et al.*, 1994) is remarkable. The two intramolecular disulphide

bridges (one in V_H and one in V_L) which are assumed to be necessary for folding into a stable and functional scFv (Glochkshuber *et al.*, 1992) are expected not to be formed in the reducing environment of the cytosol because of the absence of the enzyme protein disulphide isomerase (Freedman, 1989), which catalyzes the formation of such bonds.
Despite the reported successes, intracellular expression of scFv antibodies in plants may not be that straightforward. Owen *et al.* (1992) reported that only after screening more than 100 transgenic plants, transformed with 'leaderless' scFv constructs, a plant showing an expression level of 0.1% of the total soluble protein fraction was obtained, while transformants expressing the secretory version of the scFv gene produced ten times more scFv protein (Firek *et al.*, 1993).
The objective of our study was to compare functional expression of scFv proteins in transgenic tobacco plants if targeted to different subcellular compartments. The scFv gene was derived from the heavy and light chain genes of an antibody raised against a cutinase (21C5) of *Botrytis cinerea* (Salinas, 1992). Both with and without signal peptide the expression of this scFv gene greatly improved when the C-terminal endoplasmic reticulum (ER) retention signal peptide, KDEL (Pelham, 1989) was added. Possible causes for this strong enhancement of expression and the implications for antibody expression in plants are discussed.

Materials and methods

Bacterial vectors and strains.
For cloning of the scFv inserts the bacterial expression vector pHEN1 (Hoogenboom *et al.*, 1991) was modified by substituting the multiple cloning site and deleting the g3p gene (pNEM5). Addition of the KDEL (Lys-Asp-Glu-Leu) coding sequence behind the c-myc tag sequence resulted in pNEM5K. The *Escherichia coli* strains DH5α and TG1 were used for routine cloning and scFv protein expression, respectively.

Plant vectors.
The vectors pCPO33, pCPO33T and pCPO35 were used for plant transformations and transient assays. These vectors are closely related to pCPO5 (Florack *et al.*, 1994) and only differ between the T-DNA borders (Fig. 1). The vector pCPO33 contains a promoter-terminator cassette composed of a truncated cauliflower mosaic virus (CaMV) Cabb B-D 35S promoter (-343/-1) with duplicated enhancer sequence (-343/-90) together with the 38 base pair alfalfa mosaic virus (AlMV) RNA4 untranslated leader (Sijmons *et al.*, 1990), a polylinker with unique *Nco*I, *Sst*I, *Sma*I, and *Bgl*II cloning sites, and the nopaline synthase terminator, respectively. Furthermore, the ß-lactamase gene for prokaryotic selection (ampicillin in *E. coli*, carbenicillin in *Agrobacterium tumefaciens*) and the APH(3')II gene under the control of the nopaline synthase promoter for kanamycin resistance selection at the plant level were located between the T-DNA borders. pCPO33T contains a c-myc tag sequence (Munro and Pelham, 1986) between the multiple cloning site and nopaline synthase terminator. pCPO35 contains a mouse kappa light chain signal sequence as *Nco*I-*Sal*I fragment between the 35S promoter and the KpnI site. The mouse signal sequence for ER translocation is derived from the kappa light chain, CEA 66E3 (Kabat *et al.*, 1987, van Engelen *et al.*, 1994) and was chosen because minor changes could create a SalI site, which is rare in antibody genes (Kabat *et al.*, 1987; Chaudhary *et al.*, 1990). The signal

sequence was made synthetically with an *NcoI* site at the 5'-end (triplet position -24) and a *SalI* site at the 3'-end (triplet position -3).

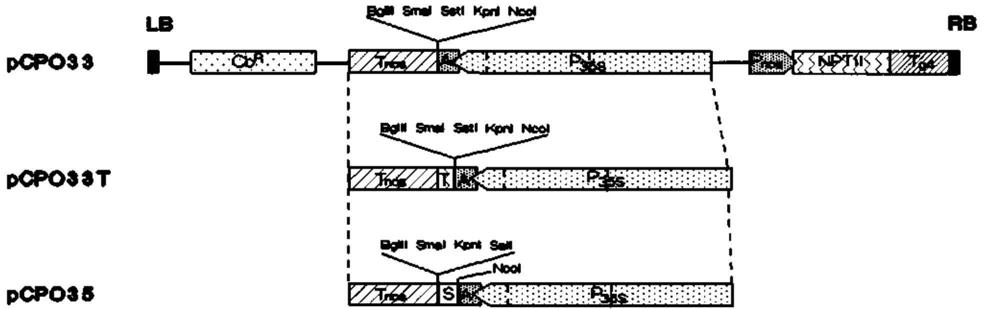

Fig. 1. T-DNA of pCPO33, pCPO33T and pCPO35. LB, left border; RB, right border; A, alfalfa untranslated leader; S, secretory signal sequence; T, c-myc tag; NPTII, neomycin phosphotransferase gene; Pnos, nopaline synthase promoer; Tg4, T-DNA gene 4 terminator; P35S, CaMV 35S promoter with doubled enhancer; Tnos, nopaline synthase terminator; Cb^R, carbenicillin resistance gene.

Isolation, amplification, and cloning of antibody 21C5 variable domains.

Isolation of poly(A^+) RNA from 21C5 hybridoma cells (Salinas, 1992) was performed by using the QuickPrep *Micro* mRNA purification kit (Pharmacia). First strand cDNA was synthesized using the Pharmacia First Strand cDNA Kit. The variable heavy (V_H) and light domains (V_L) of the 21C5 antibody were amplified through PCR using the following primers: 5'-end primer (H53) 5'-GGTCTCGAGTGTGAGGTCCAGCTGCAACAATCTG-3' and 3'-end primer (VH33) 5'-ATGCGTTAACCCCGGGTGTTGTTTTGGCTGMRGAGACDGTGAS-3' for the heavy chain, and 5'-end primer (L5d) 5'-GGTGTCGACGGTGATGTTKTGATGACCCAAA-3' and 3'-end primer (VK1) 5'-GGCTCGAGTTTGGATTCGGAGCCGGATCCTGAGGATTTACCCTCCCGTTTTAT-TTCCAGSTTGGTSCCYCC-3' for the light chain. Primers L5d and H53 contained a *SalI* and *XhoI* site at their 5'-end, respectively. Primer VH33 was chosen such that, after PCR amplification and digestion with *SmaI*, the V_H domain still contained the initial five triplets of the CH1 domain, encoding Ala-Lys-Thr-Thr-Pro. The primer VK1 carried an *XhoI* site at the 5'-end. Primer VK1 also encodes a sequence for a synthetic linker peptide, adapted with some modifications from Chaudhary *et al.* (1990). For amplification first strand cDNA was denatured at 94°C for 4 minutes and subjected to 35 cycles of PCR using Vent DNA Polymerase (New England Biolabs). Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and primer extension at 72°C for 3 min. The amplified fragments were purified from agarose gel, digested with the appropriate restriction enzymes, and ligated simultaneously into *SalI/SmaI* digested pNEM5 and pNEM5K, resulting in the vectors pNEM-scFv and pNEM-scFv-K, respectively.

The nucleotide sequences of the scFv inserts were verified by the dideoxy chain termination sequencing method (Sanger *et al.*, 1977) on an A.L.F. DNA sequencer (Pharmacia). The sequence encoding the 21C5 scFv was subjected to computer analysis with the Wisconsin GCG software package (Devereux *et al.*, 1984). From the derived protein sequence the molecular weight was calculated and the algorithm for predicting processing sites for eukaryotic signal sequences was used (von Heijne, 1986).

Bacterial expression of scFv cassettes.
E. coli strain HB 2151 was transformed with pNEM-scFv and pNEM-scFv-K. For the scFv expression assay 5 ml 2×TY, 1% (w/v) glucose and 100 μg/ml ampicillin was inoculated with a colony containing the appropriate plasmid, and incubated at 30°C for 16 hours. Fresh medium containing 2×TY, 0.075% (w/v) glucose and 1 μg/ml ampicillin was inoculated with 1/50 volume of the bacterial culture and incubated at 30°C for 3 hours. Then the scFv synthesis was induced by adding isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 1mM and the incubation was continued for another 4 hours. The periplasmic proteins were extracted by osmotic shock (Neu and Heppel, 1965). Borate buffer was added to the periplasmic fraction to a final concentration of 0.2 M sodium borate, pH 8.0, and 0.16 M NaCl. The scFvs were purified by affinity chromatography with activated protein A Sepharose (Pharmacia) to which the anti c-myc tag 9E10 monoclonal antibody (Munro and Pelham, 1986) was covalently attached.

Cloning in plant vectors and tobacco transformation.
To generate constructs suitable for cloning in plant vectors without the signal sequence the pNEM-scFv and pNEM-scFv-K vectors were digested with *Nco*I and the ends were filled in with Klenow. After digestion with *Hinc*II at the SalI site the fragments were purified and blunt end ligated resulting in the vectors pNEM-scFv-C and pNEM-scFv-CK, respectively. Thus, the *Nco*I site was restored providing the ATG start codon in the proper reading frame. Furthermore, the ATG start codon was placed at position -3 of the mature scFv sequence. The constructs lacking the KDEL sequence were cloned as SalI/SmaI (pNEM-scFv) or *NcoI/Sma*I (pNEM-scFv-C) fragments into the *NcoI/Sma*I digested plant vector pCPO33T. For construction of the scFv-S the *NcoI/Sal*I signal sequence fragment was also included in the ligation mixture. The resulting vectors, pCPO-scFv-S and pCPO-scFv-C, had the single chain construct in frame with the c-myc tag sequence. The scFv-K and scFv-CK constructs were cloned as *SalI/Bcl*I (pNEM-scFv-K) and *NcoI/Bcl*I (pNEM-scFv-CK) fragments and transferred to the *SalI/Bgl*II digested pCPO35 and *NcoI/Bgl*II digested pCPO33, respectively. The resulting vectors were designated as pCPO-scFv-SK and pCPO-scFv-CK. All vector-scFv junctions were verified by sequencing.
Tobacco transformation was conducted according to van Engelen *et al.* (1994).

Protoplasts.
Transient expression assays in tobacco (*N. tabacum* cv. Samsun NN) leaf protoplasts were performed according to the polyethylene glycol procedure as described by Denecke *et al.* (1989). The same protoplasts isolation and culture method was employed to study secretion and retention in transgenic plants. Protoplasts and culture medium were separated by centrifugation and analyzed by Western blotting experiments and ELISA. For Western analysis the proteins present in the culture medium were precipitated with 3 volumes of ethanol. Both protein pellet and protoplasts were dissolved in SDS-PAGE sample buffer (see: protein extraction and analysis). For ELISA the culture medium was diluted 1:1 with PBS-0.1% Tween-1% skimmed milk powder and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc SC, Boehringer) and the protoplasts were lysed in the same buffer. All samples were further treated as described in protein extraction and analysis.

RNA extraction and analysis.

For extracting total RNA from plant tissues the guanidine hydrochloride procedure of Logemann *et al.* (1987) was used. The RNA concentration was measured spectrophotometrically and Northern analysis was carried out according to Sambrook *et al.* (1989).

Briefly, 9 μg RNA was separated on a 1.2% (w/v) agarose (Pharmacia) formaldehyde gel. As size marker 1 ng denatured 21C5 scFv DNA and 1 μg of the 0.16-1.77 kb RNA ladder (Life Technologies) were used. After electrophoresis the gel was incubated twice for 15 minutes in DEPC treated double distilled water and the RNA was transferred to a Hybond-N+ membrane (Amersham) by vacuum blotting, using 20×SSC as transfer buffer, and cross-linked to the membrane under UV light at 1.5 J/cm^2. The blot was hybridized with [α-^{32}P]dATP-labeled probes at 65°C for 48 hours and further treated as described by Church and Gilbert (1984). The stringency of the final washing was 0.2×SSC at 65°C. The blot was first hybridized with a labeled scFv DNA fragment, isolated as SalI-SmaI fragment from pNem-scFv. To establish the differences in the amount of total RNA the blot was hybridized with a ribosomal probe. To estimate the molecular sizes the blot was hybridized to labeled cDNA of the RNA ladder. All probes were obtained by random prime labeling (Feinberg and Fogelstein, 1983).

Protein extraction and analysis.

Proteins were extracted by grinding tobacco leaves in liquid nitrogen to a fine powder. The powder was transferred to an eppendorf tube and mixed 1:1 (w/v) with SDS-PAGE sample buffer, containing 61 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 12.5% (w/v) glycerol, 1 mM Pefabloc SC (Boehringer). Insoluble plant material was pelleted by centrifugation for 5 minutes at 13,000 rpm. The protein concentration in the supernatant was determined using the BCA method (Smith *et al.*, 1985). To the supernatant DTT and bromophenolblue were added to final concentrations of 40 mM and 0.008% (w/v), respectively, and the samples were boiled at 100°C for 5 minutes. For non-reducing gel electrophoresis DTT was omitted during sample preparation. Thirty μg of total protein was loaded on a 13% SDS-polyacrylamide gel Laemmli, 1970) (Bio-Rad mini protean system). After electrophoresis the proteins were transferred to a PVDF membrane (Millipore) by electroblotting. For immunodetection the membranes were incubated with 1:1000 diluted 9E10 monoclonal antibody, followed by a 1:5000 diluted rat-anti-mouse alkaline phosphatase conjugate (Jackson Immuno Research). Alternatively a rabbit polyclonal anti-21C5 serum, precleared from antibodies reacting to the constant domains, was used in conjunction with a 1:2500 diluted goat-anti-rabbit alkaline phosphate conjugate (Jackson Immuno Research). The blots were stained in 0.1 M ethanolamine-HCl pH 9.6, supplemented with 4 mM $MgCl_2$, 5-bromo-4chloro-3-indolyl phosphate (0.06 mg/ml) and nitro blue tetrazolium (0.1 mg/ml). The relative molecular weights of the proteins were estimated with pre-stained low range molecular weight markers (Bio-Rad).

Purification of native scFv 21C5 antibody from plant extracts was carried out by polytron homogenization of 4 g tobacco leaves in 4 ml 0.2 M sodium borate, pH 8.0, containing 0.16 M NaCl and 1mM Pefabloc SC (Boehringer), in the presence of 200 mg insoluble polyvinylpyrrolidone (Serva). The soluble protein fraction was isolated by centrifugation and filtered through a 0.45 μm filter (Millipore). The scFvs were purified by affinity

A

```
scFV-S          DVVMTQ==(VL)==IKREGKSSGSGSESKLECEV==(VH)==AAKTTPGRSEQKLISEEDLN
scFV-C        DGDVVMTQ==(VL)==IKREGKSSGSGSESKLECEV==(VH)==AAKTTPGRSEQKLISEEDLN
scFV-SK         DVVMTQ==(VL)==IKREGKSSGSGSESKLECEV==(VH)==AAKTTPGAAAEQKLISEEDLNDIKDEL
scFV-CK      MDGDVVMTQ==(VL)==IKREGKSSGSGSESKLECEV==(VH)==AAKTTPGAAAEQKLISEEDLNDIKDEL

Ec-scFv    QVDGDVVMTQ==(VL)==IKREGKSSGSGSESKLECEV==(VH)==AAKTTPGAAAEQKLISEEDLNDI
Ec-scFv-K  QVDGDVVMTQ==(VL)==IKREGKSSGSGSESKLECEV==(VH)==AAKTTPGAAAEQKLISEEDLNDIKDEL
```

B

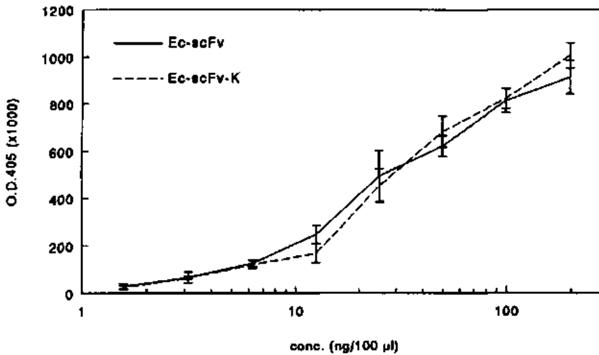

Fig. 2. A. Predicted amino acid sequence of the different mature scFv antibody constructs expressed in plants and bacteria. The V_L and V_H domains are connected by a 16 amino acid linker peptide (bold). The c-myc tag (underlined) is located at the C-terminal end of the peptide. B. Antigen-binding activity of scFv antibodies, isolated from *E. coli*, assayed by ELISA using the anti c-myc antibody. Serial dilutions of purified Ec-scFv and Ec-scFv-K antibodies were incubated in wells coated with 100 ng cutinase. Individual points represent mean values of triplicate trials with standard deviations (error bars).

chromatography with the 9E10 monoclonal antibody coupled to activated protein A Sepharose (Pharmacia) as described previously.
For use in ELISA assays the proteins were extracted by grinding 0.2-0.4 g tobacco leaves in liquid nitrogen to a fine powder. The powder was transferred to an eppendorf tube and mixed 1:2 (w/v) with PBS-0.1% (v/v) Tween (PBST) and 1 mM Pefabloc SC (Boehringer), and incubated on ice for 5 minutes. Insoluble material was removed by centrifugation at 13.000×g. The supernatant was stored at -80°C until further use.
The cutinase binding activity of the crude supernatant or purified scFv was determined by ELISA. A 96-well plate was coated overnight with 1 µg/ml cutinase in 50 mM sodium carbonate, pH 9.6 (100 µl/well). After blocking for 30 minutes with 200 µl PBST-5% skimmed milk powder per well the plates were washed and 100 µl protein extract per well was added. The plate was incubated for 2 hours. To determine the antigen binding capacity of the scFv antibody preparations, the wells were subsequently washed with PBST, eluted with SDS-PAGE sample buffer, and analyzed by immunoblotting under non reducing conditions. Alternatively, for quantitative ELISA, after washing with PBST each well was incubated for another two hours with 100 µl anti c-myc tag antibody 9E10 (1 ng/µl) in PBST-1% skimmed milk powder. Then, after washing three times with PBST, the wells were incubated for 1 hour with alkaline phosphatase conjugated rat-anti-mouse antibody (Jackson Immuno Research), diluted 1:5000 in PBST-1% skimmed milk powder. Finally the wells were washed five times

with PBST and 100 µl substrate (0.75 mg/ml p-nitrophenylphosphate in 1M diethanolamine, pH 9.8) was added and the OD_{405} was monitored. All incubations were carried out at 37°C.

Results

Construction of the scFv expression cassettes.

An scFv gene was constructed containing the variable domains of the 21C5 antibody heavy (V_H) and light chain (V_L) genes (van Engelen *et al.*, 1994) in the 5'-V_L-linker-V_H-3' orientation. The end of the V_H region was fused to the c-myc tag coding sequence for detection and purification purposes. To enable translocation of the 21C5 scFv to the lumen of the ER it was preceded by a murine κ light chain signal peptide (scFv-S; Fig. 2A). This signal peptide has shown previously to export full-size antibodies efficiently to the plant apoplast (van Engelen *et al.*, 1994). To retain the scFv-S antibody in the ER a C-terminal ER retention signal KDEL was added (scFv-SK; Fig. 2A). In addition two cytosolic versions of the 21C5 scFv (scFv-C and scFv-CK; Fig. 2A) were constructed, which both lacked the ER translocation signal.

To determine if the presence of the KDEL retention signal had any effect on either antigen binding capacity or detection with the anti-c-myc tag antibody, the scFv genes, with and without KDEL sequence, were expressed in *E. coli* (Ec-scFv-K and Ec-scFv, respectively; Fig. 2A). Both scFv genes were preceded by the pelB signal peptide. After affinity purification the Ec-scFv and Ec-scFv-K antibodies showed similar binding properties to the cutinase antigen in an ELISA assay (Fig. 2B). Western blotting followed by immunodetection using the anti c-myc tag antibody revealed proteins of 31 kDa (Fig. 3). Apparently, addition of the KDEL retention signal had no effect on the binding properties of the anti c-myc tag antibody 9E10.

Expression of scFv antibodies in transgenic tobacco leaves.

The scFv cassettes were introduced into tobacco by *Agrobacterium* mediated transformation. As a control, transformation also was conducted using the empty vector pCPO33T. Independent kanamycin resistant transformants were screened by immunoblotting of total protein extracts from leaves. All 43 plants containing the scFv-S constructs showed poor expression. By comparison of the staining intensity on Western blot of the plant produced scFv protein with known amounts of bacterially produced scFv, it was estimated that the maximum expression level reached was 0.01% of total protein. No scFv protein was detected in 23 plants containing the scFv-C construct. However, 9 out of 15 scFv-CK transformants showed scFv antibody expression with a maximum level of 0.2% (Fig 3). From the 15 scFv-SK transformants 13 were expressing scFv protein, the highest expression level being 1.0% (Fig. 3).

In plants expressing the scFv-SK protein an additional minor product of approximately 65 kDa was detected. To gain more insight in the nature of this 65 kDa band, protein samples were prepared from leaves and analyzed under non-reducing conditions. Western blotting showed that under these circumstances the fraction of the 65 kDa protein increased considerably for both scFv-SK and scFv-CK protein preparations (Fig. 4A). This could indicate that in plant cells the cysteine residue present in the linker peptide (Fig. 2A) may have been involved in dimer formation. To determine if both scFv protein and the presumed

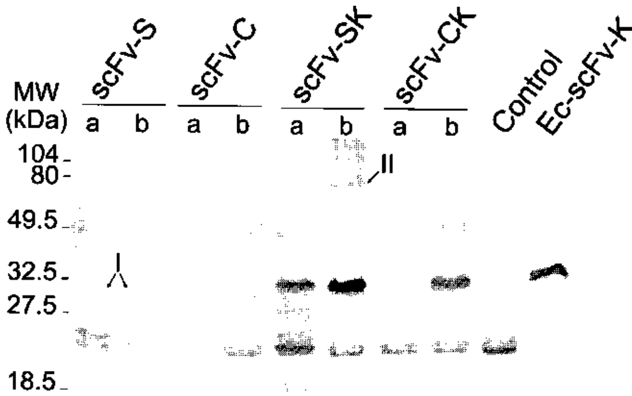

Fig. 3. Western blot analysis of leaf tissue from two independent tobacco transformants (a and b) containing the scFv-S, scFv-C, scFv-SK and scFv-CK cassettes. The lanes marked 'Control' are from a transgenic tobacco plant transformed with the vector pCPO33T. The scFv antibodies were detected using the anti-c-myc antibody (9E10). The arrows marked 'I' indicate the scFv-S antibody and the arrow marked 'II' indicates the 65 kDa protein band.

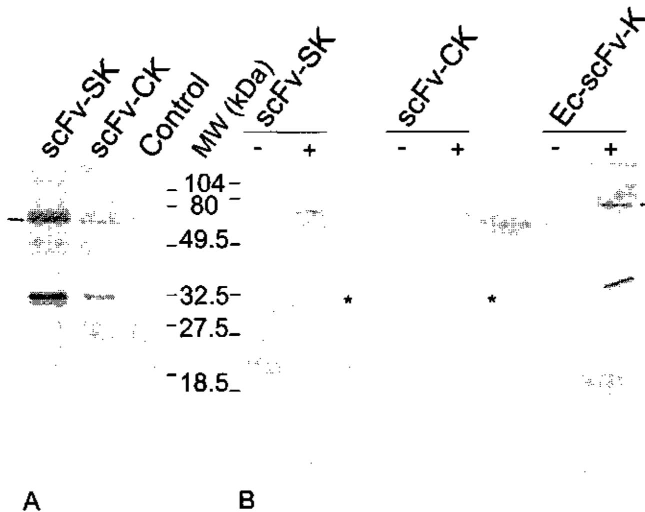

Fig. 4. A) Western blot of proteins from scFv-SK and scFv-CK transgenic tobacco after non-reducing electrophoresis. The scFv antibodies were detected using the anti-c-myc antibody (9E10). Arrow indicates the 65 kDa protein band. B) Binding of scFv antibodies to cutinase. Immunoblot of proteins eluted from wells coated with (+) or without (-) cutinase after incubation with scFv-SK and scFV-CK antibodies purified from plants and the scFv-K antibody purified from *E. coli*. The antibodies were detected with the anti-c-myc antibody (9E10). Asterisk and arrow indicate the scFv antibodies and 65 kDa protein bands, respectively.

scFv-dimer had antigen binding capabilities, purified scFv-CK and scFv-SK antibodies were incubated with immobilized cutinase and analyzed after elution under non-reducing conditions (Fig. 4B). Purified bacterially expressed Ec-scFv-K was used as a control. Western blotting of the eluents showed that not only scFv-CK and scFv-SK monomers, but also the 65 kDa proteins bound specifically to the cutinase antigen.

Accumulation of scFv mRNA and protein in transgenic tobacco leaves.

Since the KDEL retention signal is thought to function only in the secretory pathway the difference in expression level between the scFv-C and scFv-CK was a surprise. Therefore, we investigated whether the differences in protein accumulation between the various constructs could be explained by differences in the steady state mRNA levels. For a number of plants both total RNA and protein was isolated from the same leaf and analyzed (Fig. 3 and 5).

Northern blot analysis showed that the scFv transgenic plants accumulated scFv mRNA of the expected size of 1000-1200 bases, albeit in different quantities (Fig. 5A). In addition, a much less abundant mRNA of 1400 bases was detected. The origin of this mRNA is not

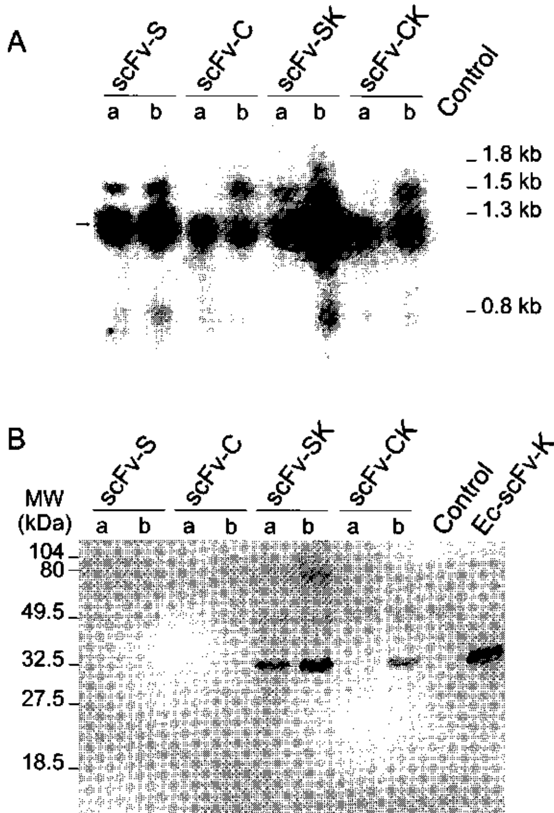

Fig. 5. RNA and protein analysis of leaf tissue from the same independent tobacco transformants as depicted in Fig. 3. A. RNA blot containing total RNA hybridized with a specific scFv probe. Arrow indicates position of scFv transcripts. B. ScFv antibodies detected on Western blot using the polyclonal rabbit antiserum against the 21C5 antibody.

clear. It was not detected in control plants and therefore may be a read-through product of the scFv messenger RNA. The difference in protein expression level between the different scFv genes could not be explained by various levels of scFv mRNA. The scFv-C mRNA level (Fig. 5A: lane a) was comparable with the scFv-CK mRNA level (Fig. 5A: lane b) but no scFv-C protein was present whereas scFv-CK protein was detected (Fig. 3 and 5B: lanes a and b). Furthermore, a low scFv-SK mRNA level (Fig. 5A: lane a) resulted in a higher scFv protein accumulation than the relative high scFv-S mRNA level (Fig. 5A: lanes a and b).
To exclude the possibility that the c-myc tag had been removed by plant proteases, thereby affecting our detection procedure, we used both anti-tag antibodies (Fig. 3) and an anti-21C5-Fv rabbit polyclonal antiserum (Fig. 5B) for scFv detection in a number of transgenic plants. Essentially the same results were obtained, indicating that the presence of the complete scFv antibody correlated with the presence of the tag.
The addition of the KDEL retention signal elevated the steady state levels of the 21C5 scFv antibody, both with and without signal peptide.

Expression of the scFv antibodies in tobacco leaf protoplasts.
The four different mature scFv proteins varied slightly in their number of amino acids (Fig. 2A). The calculated sizes of the mature scFv proteins ranged from 30 kDa for the scFv-S to 31 kDa for the scFv-CK. An uncleaved signal peptide would increase the calculated size for the scFv-S and scFv-SK antibodies by 2.5 kDa. On Western blot the protein bands showed only minor size differences, the smallest molecule being the scFv-S protein (Fig. 3). This might indicate that the signal peptides of both scFv-S and scFv-SK proteins were recognized and cleaved off during translocation into the ER.
To determine whether the signal peptide and the KDEL retention signal had the predicted effects on scFv protein translocation, transient expression assays were carried out in

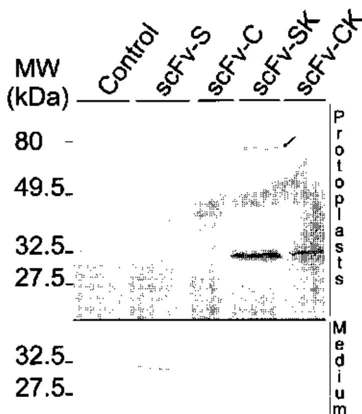

Fig. 6. Western blot analysis of a transient expression assay in tobacco protoplasts transformed with plant vectors containing the scFv-S, scFv-C, scFv-SK and scFv-CK gene cassettes. The 'Control' lane represents the transformation of tobacco protoplasts with the vector pCPO33T. The scFv antibodies present in the cells and incubation medium were detected by the anti-c-myc antibody (9E10). Arrow indicates the 65 kDa protein band.

tobacco protoplasts. Western blot analysis showed differences in the location of the scFv proteins (Fig. 6). As expected the scFv-S protein was secreted into the incubation medium indicating that the signal peptide was indeed functional. The scFv-SK and scFv-CK proteins were predominantly found inside the protoplasts. The residual presence of scFv-CK and scFv-SK protein in the incubation medium was probably due to cell disruption during the assay, since in a control experiment expressing a β-glucoronidase (GUS) construct lacking a signal peptide some GUS activity was detected in the medium. From the data obtained with the scFv-SK expression we concluded that the KDEL retention signal was able to retain the scFv-SK antibody inside the protoplasts. This was confirmed by using protoplasts, prepared from the transgenics with a high scFv mRNA level, which showed only intracellular accumulation of the scFv-SK. However, in this case we could not detect the scFv-S antibody, neither in the protoplasts nor in the medium (results not shown).

Discussion

Successful applications for scFv antibodies expressed in plants, including creating resistance against pathogens (Tavladoraki *et al.*, 1993) or altering metabolic pathways (e.g. catalytic antibodies), will to a large degree depend on the ability to target the scFvs to a particular subcellular compartment and to optimize their expression level. Tavladoraki *et al.* (1993) described the successful expression of an scFv antibody directed to the cytosol. Firek *et al.* (1993) reported a significant increase in the expression level of an scFv antibody against phytochrome when secreted instead of expressed in the cytosol (Owen *et al.* 1992). They suggested that this difference in expression levels was not the result of a difference in subcellular location but was caused by a destabilizing effect of the phytochrome on the cytosolic scFv (Firek *et al.*, 1993).
Since no further data on the expression of scFv antibodies in different subcellular compartments of plant cells were available we decided to explore the possibilities of intracellular targeting of scFv antibodies and assess the effect on stability and accumulation. To improve intracellular stability we targeted an scFv antibody away from the cytosol to the potentially more favourable environment of the endoplasmic reticulum (ER) by adding a signal peptide and the tetrapeptide KDEL (Herman *et al.*, 1990; Denecke *et al.*, 1992; Wandelt *et al.*, 1992) (scFv-SK). For comparison a secretory version (scFv-S) of this molecule was used, as well as two cytosolic counterparts, one with and one without the KDEL retention signal (scFv-CK and scFv-C, respectively). The expression level and localization of this scFv-SK antibody were compared with those of the scFv-C, scFv-CK, and scFv-S antibodies.
Of the tobacco transformants expressing the scFv-SK cassette, 85% showed a high accumulation of the protein in leaves. In some plants the scFv protein comprised up to 1% of the total soluble protein. Protoplasts prepared from these transgenic plants showed total retention of scFv-SK in the cells. This was confirmed by transient expression assays in tobacco protoplasts. The ScFv-SK antibody was retained intracellularly while a large proportion of the scFv-S antibody was secreted into the culture medium. These results indicated that the signal peptide was functional. Furthermore, they showed that the KDEL retention signal was probably well exposed, recognized by a salvage receptor (Vaux *et al.*, 1989; Tang *et al.*, 1994), thereby enabling the scFv antibody to be retained in the ER. When compared with the plants expressing the secreted scFv (scFv-S) the retention in the ER

resulted in a 100-fold increase in the amount of detectable scFv antibody. These high accumulation levels cannot be explained by differences in the mRNA levels. It therefore seems that the high level of scFv antibody accumulation is due to its strict localization in the ER and consequently is protected from proteolytic activity further down the secretory pathway, either intra- or extracellularly. Similar results have been obtained with the vacuolar protein vicilin, which also accumulated to a much higher level when retained in the ER (Wandelt *et al.*, 1992).

Most striking were the differences in expression levels obtained with the scFv-C and scFv-CK constructs. No transgenic tobacco plants could be found with detectable levels of scFv-C antibody. In contrast to this finding, among the scFv-CK transformants 60% of the plants showed detectable antibody levels. In one plant the scFv-CK protein level reached 0.2% of the total soluble protein, which is comparable with previously reported cytosolic expression levels (Owen *et al.*, 1992; Tavladoraki *et al.*, 1993). This difference in expression between the two constructs (scFv-C and scFv-CK) was also found in the transient expression assay. The steady state levels of scFv mRNA indicated that the difference in protein accumulation most likely depended on differences in stability of the protein. This phenomenon is not unique for the anti-cutinase scFv, since we have recently obtained similar results with another scFv antibody (unpublished results).

Presently we can only speculate on the factors that cause these KDEL correlated differences in expression in plants. Assuming that both scFv-C and scFv-CK antibodies are located in the cytosol, it might be possible that the C-terminal extension of the scFv-CK antibody, which is in fact six amino acids long (DIKDEL), protects the scFv from C-terminal degradation by exo-proteinases. This then would suggest that particular exo-proteinases are involved in the breakdown of scFvs. Alternatively, the DIKDEL sequence may indirectly protect the scFv from proteolytic attack via a KDEL mediated interaction with the cytosolic side of the ER salvage receptor (Tang *et al.*, 1994).

Another explanation for the observed differences could be that expression levels of the scFv are correlated to a different subcellular location. It has been well documented that the expression of normally secreted proteins, particularly those with disulphide bridges, in the cytosol of plant cells is very low (Sijmons *et al.*, 1990; Bosch *et al.*, 1994; Florack *et al.*, 1994). It is therefore not surprising that the scFv-C transformants failed to produce detectable amounts of scFv antibodies. Protein analysis using the algorithm for predicting signal peptidase cleavage sites (von Heijne, 1986) within the GCG Wisconsin program revealed that both mature scFv-C and scFv-CK proteins did not contain a signal peptide-like sequence in the amino-terminal region. Possibly, the KDEL containing scFvs, even when no signal peptides are added, are directed away from the cytosol to a more favourable location, presumably the ER. The presence of substantial amounts of the 65 kDa protein in the scFv-CK transgenic plants along with its functionality might indicate an ER location. Noteworthy in this respect is the recent suggestion that scFv antibodies targeted to the cytosol of animal cells were actually "mistranslocated" to the ER (Jiang *et al.*, 1995). In addition, alternative pathways for secretory proteins, lacking signal peptides, have been put forward (Muesch *et al.*, 1990).

The very low expression from the scFv-S construct in transgenic plants and transgenic protoplasts contrasts with the result obtained in transient expression experiments where we could detect the scFv-S extracellularly. Possibly the protoplasts used for the transient assay were physiologically different from the transgenic scFv-S protoplasts and produced less proteases into the incubation medium. Firek *et al.* (1993) reported high expression levels in

plants when an anti-phytochrome scFv antibody was being secreted. This difference in stability between different scFv antibodies is not clear but may depend on the amino acid constitution in the variable domains of the scFv antibodies or the linkerpeptide.
Efficient expression of scFv antibodies in different subcellular sites seems feasible. However, it should be kept in mind that successful expression of functional scFvs in the cytosol may only be found under certain conditions, like an scFv amino acid sequence which remains relatively stable (Tavladoraki *et al.*, 1993) or at least can be stabilized by the presence of the antigen (Biocca *et al.*, 1990; Firek *et al.*, 1993). The C-terminal addition of the retention sequence KDEL as a contributing factor for scFv stabilization opens additional opportunities for expressing scFv antibodies in plants.

Acknowledgements

We would like to thank Dr Jesús Salinas for providing the anti-cutinase hybridoma cell line and Jan de Boer for critical reading of the manuscript and helpful discussion. This work was financially supported by grants from the Netherlands Technology Foundation (STW), coordinated through the Foundation for Life Sciences (SLW), and the European Commission (BIO2-CT92-0239).

CHAPTER 4

Improving scFv antibody expression levels in the plant cytosol

This chapter was published in *FEBS Letters* **415** (1997), 235-241, by the authors A. Schouten, J. Roosien, J.M. de Boer, A. Wilmink, M.-N. Rosso, D. Bosch, W.J. Stiekema, F,J. Gommers, J, Bakker and A. Schots.

Abstract

Expression of single-chain antibody fragments (scFvs) in the plant cytosol is often cumbersome. It was unexpectedly shown that addition at the C-terminus of the ER-retention signal KDEL resulted in significantly improved expression levels. In this report the cytosolic location of the scFv-CK was confirmed, excluding possible mistranslocation to other subcellular compartments. It was shown that expression of several other scFvs was also improved in tobacco protoplasts. In addition expression was improved in transgenic potato. Changing from KDEL into KDEI did not affect the enhanced protein expression level. Addition of the KDEL motif is a simple and straightforward tool to stabilize *in planta* cytosolic expression of many scFvs.

Introduction

The expression of specific antibodies or antibody fragments in plant cells to obtain phenotypic mutants, like resistance against pathogens (Tavladoraki *et al.*, 1993; Voss *et al.*, 1995) or altered metabolic pathways (Owen *et al.*, 1992; Artsaenko *et al.*, 1995), has proven its potential. The use of single-chain Fv fragments (scFvs), consisting of the variable heavy and light chain domains coupled by a linker peptide, is of particular interest since these molecules were functionally expressed in both the secretory pathway and ectopic environments. Even in the reducing environment of the plant cytosol scFvs can fold into functional molecules. This folding capability has also been demonstrated in *Xenopus* oocytes, COS cells and human cells (Biocca and Cattaneo, 1995; Duan *et al.*, 1994).
Despite these successes, cytosolic expression of scFv antibodies in plants is often low or absent (Fiedler *et al.*, 1995; Bruyns *et al.*, 1996; Schouten *et al.*, 1996; Whitelam and Cockburn, 1996; Fecker *et al.*, 1997). If the stability in the cytosol is an intrinsic property of the variable domains it will be a tedious job to improve expression levels for each individual scFv antibody without loosing affinity. It would therefore be more convenient to search for a general approach to stabilize cytosolic scFv molecules without modifying the variable domains.
Recently, we obtained a significantly improved stability in tobacco when the tetrapeptide KDEL was added at the C-terminal end of an anti-cutinase scFv (scFv-CK) designed to be located in the cytosol (Schouten *et al.*, 1996). This tetrapeptide is the signal for retaining proteins in the endoplasmic reticulum (ER) when translocated into the secretory pathway (Pelham, 1989; Denecke *et al.*, 1992; Wandelt *et al.*, 1992; Artsaenko *et al.*, 1995). The addition of a four amino acid extension may be a valuable approach to stabilize cytosolic scFvs without modifying the variable domains.
In this report we first established that scFv-CK was present in the cytosol of tobacco, to exclude possible mistranslocation to the ER, reported by (Jiang *et al.*, 1995), as a cause for improved scFv protein expression levels. We then investigated if the tetrapeptide KDEL could also be beneficial for the cytosolic expression level of several other scFv antibodies and be successfully applied for the expression of scFvs in another plant species, potato. Finally, to study whether or not the tetrapeptide could be slightly modified without loosing its positive effect on protein stability in the cytosol, the leucine of the tetrapeptide KDEL of one of the scFvs was changed into isoleucine and this antibody fragment was expressed in potato.

Materials and methods

Cell lines, vectors and strains.

Single chain antibodies were constructed starting from the hybridoma cell lines MGR48, MGR49 and MGR59 (de Boer *et al.*, 1996), all producing monoclonal antibodies reacting with β-1,4-endoglucanase, and from the hybridoma line anti-GUS, which produces an anti-β-glucuronidase monoclonal antibody.

For construction of the various 21C5 anti-cutinase scFv genes the vectors pNEM-scFv, pNEM-scFv-K and pCPO-scFv-CK (Schouten *et al.*, 1996) were used.

For transient expression assays scFv antibodies were cloned in pRAP-scFv-SK, pTR2-scFv-SK or pTR2-scFv-S. To obtain pRAP-scFv-SK the vector pUCAP35S (van Engelen *et al.*, 1995) was provided with a kappa signal peptide (van Engelen *et al.*, 1994) and the scFv cassette with carboxy terminal KDEL extensions, obtained from the vector pNEM-scFv-K (Schouten *et al.*, 1996). The thus obtained vector pRAP-scFv-SK can accept other scFv encoding genes as *SalI*/*NotI* fragments between the kappa signal peptide and the c-myc tag plus C-terminal KDEL extension. To obtain pTR2-scFv-SK and pTR2-scFv-S we first cloned the 1.4 kb *Hind*III fragment from pCPO5 (Florack *et al.*, 1994) containing the 35S terminator (T35S), the TR2'-1' dual promoter and octopine synthase terminator (Tocs) into the *Hind*III site of pAP (van Engelen *et al.*, 1995). Then the original *SalI* site, present between the TR1' promoter and Tocs was removed by filling in, creating the vector pTR2. The anti-cutinase scFv cassettes with the coding sequence for signal peptide and C-terminal c-myc tag, with and without KDEL retention signal, were isolated from pNEM-scFv-K and pNEM-scFv as *NcoI*/*BclI* fragments and inserted into *NcoI*/*Bam*HI digested pTR2, resulting in the vectors pTR2-scFv-SK and pTR2-scFv-S, respectively.

Construction of scFv genes with coding sequence for glycosylation in the linker.

To modify the 202' derived peptide linker (Pantoliano *et al.*, 1991) of the 21C5 anti-cutinase scFv the V_L domain was reamplified using the primers L5d (5'-GGTGTCGACGGTGATGTTK-TGATGACCCAAA-3') and Vkglyc (5'-AGCCGGATCCGTTGGATTTACCCTCGAGTTTTATTTCCAR-CTTKGTSCC-3'). Restriction sites in the primers, used in the cloning procedure, are underlined. PCR conditions were used as described by Schouten *et al.* (1996). The amplified fragment was inserted into the *SalI*/*Bam*HI restricted vector pNEM-scFv. The resulting pNEM-scFv^{glyc} contained a linker flanked by *XhoI* sites. With respect to the original scFv the Arg at position 1 of the peptide linker was replaced by Leu (Fig. 1). The Ser at position 6 was replaced by Asn, thereby creating a consensus site for N-linked glycosylation (Kornfeld and Kornfeld, 1985). A control scFv construct in which the Asn was replaced by Ser, but the Leu at position 1 retained, was obtained by inserting an *XhoI*/*Bam*HI adapter fragment (5'-TCGAGGGTAAATCCTCCG-3' and 5'-GATCCGGAGGATTTACCC-3') into *XhoI*/*Bam*HI digested pNEM-scFv^{glyc}, creating pNEM-scFv$^{\Delta glyc}$. The modified scFv constructs were cloned as *SalI*/*NotI* fragments from pNEM-scFv^{glyc} and pNEM-scFv$^{\Delta glyc}$ into pRAP-scFv-SK, creating pRAP-scFv^{glyc}-SK and pNEM-scFv$^{\Delta glyc}$-SK, respectively (Fig. 1).

The scFv gene insert from the vector pRAP-scFv-SK was replaced by the *NcoI*/*NotI* scFv insert from pCPO-scFv-CK, resulting in the vector pRAP-scFv-CK. To obtain scFv-CK constructs with identical linker peptide coding sequences with and without glycosylation signal the *PstI* fragment in this vector was replaced with the same insert from the vectors

pRAP-scFv^{glyc}-SK and pRAP-scFv$^{\Delta glyc}$-SK. The resulting constructs were verified on proper orientation and subsequently called pRAP-scFv^{glyc}-CK and pRAP-scFv$^{\Delta glyc}$-CK, respectively (Fig. 1).

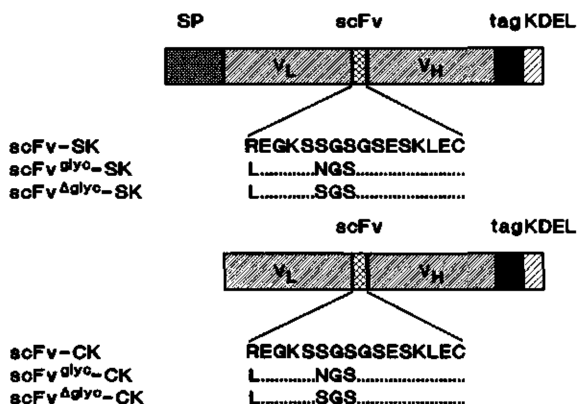

Fig. 1. Diagram of the scFv-SK and scFv-CK constructs with modifications in the linker peptide. The signal for N-linked glycosylation, Asn-X-Ser, was introduced by changing the Ser of both scFv$^{\Delta glyc}$-SK and scFv$^{\Delta glyc}$-CK into Asn, resulting in the sequence Asn-Gly-Ser in the constructs scFv^{glyc}-SK and scFv^{glyc}-CK, respectively. The N-terminal signal peptide (SP), the single chain antibody construct (scFv) with the variable the variable light (V_L) and heavy (V_H) chain domain connected by the linker peptide, the C-terminal c-myc tag (tag) followed by the KDEL peptide sequence are indicated.

Immunocytolocalization.

Young leaves (length approximately 3 cm) of *in vitro* grown transgenic tobacco plants, expressing the anti-cutinase scFv-CK and scFv-SK antibodies, were submersed in a solution of 4% formaldehyde, 0.1 M HEPES-NaOH pH 7.5 and 1.5 mM $CaCl_2$. The leaf tissue was cut into strips of approximately 1 × 5 mm, which were degassed briefly *in vacuo* and left to fix overnight at room temperature. Following washing in water, the leaf strips were dehydrated in ethanol and embedded in LR-Gold resin following standard low-temperature procedures (Vandenbosch, 1991). Immunogold labeling of thin sections was performed essentially as described by de Boer *et al.* (1996), using monoclonal antibody 9E10 to the c-myc tag at a concentration of 1 μg/ml as the primary antibody, and a 10 nm goat-anti-mouse gold conjugate (Aurion, Wageningen, The Netherlands) for detection. Control labeling experiments were performed with leaf tissue from untransformed plants, and by omitting the primary antibody in the labeling procedure.

Construction of scFv antibody genes.

Isolation of poly(A)$^+$ RNA from hybridoma cells was performed using the QuickPrep Micro mRNA purification kit (Pharmacia). cDNA was synthesized using the Pharmacia First Strand cDNA Kit.

To create the scFv48 gene the MGR48 cDNA was amplified by PCR using the primer combination L5d-Nco (5'-CGTGCCATGGATGTTKTGATGACCCAAACTC-3') and 202VL3 (5'-GG-ATTCAGATCCGGATCCTGAGGACTTACCCTCGAGCTTTATTTCCAGCTTGGTCCC-3') for the V_L domain and 202VH5 (5'-TCAGGATCCGGATCTGAATCCAAGCTCGAGTCTCAGGTCCAGTTGGT-ACAGTCTG-3') and VH33b (5'-GCACGTTAACCCCGGGTGTTGTTTTGGCTGCAGAGACAG-3') for the V_H domain. The *NcoI/Bam*HI digested V_L and *Bam*HI/*Hpa*I digested V_H fragments were ligated together in the *NcoI/SmaI* digested pTR2-scFv-S and pTR2-scFv-SK.
For the scFv49 construction we amplified MGR49 cDNA using primer combination L5h (5'-GGTGTCGACGGTGACATCCAGATGACMCAGWCTMCM-3') and 3KGS to obtain V_L and 5HGS (5'-GGTGGAGGATCCGGTGGAGGAGGTTCTGAGGTYCAGCTGCARSA-3') and VH34 (5'-ATGC-GTTAACCGTTGTTTTGGCTGMRGAGACDGTGAS-3') to obtain V_H. The VL and VH domains were fused using splicing by overlap extension (SOE) (Horton *et al.*, 1990) and initially ligated as a *SalI/HpaI* fragment into pNEM6 (Rosso *et al.*, 1996). Finally the scFv49 fragment was cloned as *NcoI/NotI* fragment into *NcoI/NotI* digested pTR2-scFv-S and pTR2-scFv-SK.
To create scFv59 MGR59 cDNA was amplified with the primers L5d-Nco and 3Kgs2 (5'-CG-CCTCCGGAGCCTCCACCACCGGAACCACCACCACCGGATCCCCCTTTTATTTCCARCTTKTGTC-3') for the V_L domain and gsH5c (5'-GGTGGAGGCTCCGGAGGCGGAGGATCCGAGGTCCAGC-TGCAACARTC-3') and VH33a (5'-GCACGTTACCCCGGGTGTTGTTTTGGCTGAGGAGACKG-3') for the V_H domain. After *NcoI/Bsp*EI and *Bsp*EI/*SmaI* digestion the domains were ligated together in the *NcoI/SmaI* digested pTR2-scFv-S and pTR2-scFv-SK.
To create scFvGUS anti-GUS cDNA was amplified using the primer combination L5h and VLK2 (5'-GACTCGAGTTTGGATTCGGAGCCGGATCCTGAGGATTTACCCTCCCGTTTTATTTCCA-RCTTKGTCCCMG-3') to obtain V_L and primer combination FVH3 (5'-TCAGGATCTGGCTC-CGAATCCAAACTCGAGTCTGAGGTGAAGCTGGTGGARTCTG-3') and VH33 to obtain V_H. The VL and VH domains were fused by SOE and initially ligated as a *SalI/SmaI* fragment into pNEM5 (Schouten *et al.*, 1996). Then the scFvGUS gene was ligated as *NcoI/NotI* fragment into *NcoI/NotI* digested pRAP-scFv-S and pRAP-scFv-SK.
Cloning procedures were according to Sambrook *et al.* (1989). All constructs were checked by sequencing (Sanger *et al.*, 1977). The sequence data were deposited in the GenBank database under the accession numbers AF004403; AF004404, AF00440, AF004406 and AF004407.

Transient expression and protein analysis.
Transient expression assays in tobacco (*N. tabacum* cv. Samsun NN) leaf protoplasts were performed essentially according to the polyethylene glycol procedure as described by (Denecke *et al.*, 1989). In case of tunicamycin treatment the transfected protoplast were incubated in TEX medium supplemented with 10 μg/ml tunicamycin (Iturriaga *et al.*, 1989).
The protoplasts were separated from the incubation medium and lysed by adding an equal volume of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 1 mM Pefabloc SC (Boehringer), and subsequent vortexing. The chloroplasts were pelleted by centrifugation at 13000×g for 2 min and the supernatant was used for further analysis. For western analysis one third volume of 4×SDS-PAGE sample buffer, containing 244 mM Tris-HCl, pH 6.8, 8% (w/v) SDS, 50% (w/v) glycerol and 1 mM Pefabloc SC (Boehringer), was added to an aliquot of the supernatant, equaling 3×10^4 protoplasts. The samples were incubated at 100°C for 5 min and bromophenolblue was added to a final concentration of 0.008% (w/v).
For deglycosylation proteins isolated from 8×10^4 protoplasts in a final volume of 20 μl, were denatured by adding 2.2 μl 5% (w/v) SDS, 2% (v/v) β-mercaptoethanol and incubation at

100°C for 5 minutes. The sample was split into two portions. Both aliquots received 2 μl 0.5 M sodium phosphate, pH 7.5, 3 μl water and 2 μl 10% (v/v) NP-40. To the first aliquot 2 μl PNGase F (500 units/μl, New England Biolabs) was added. As a control, 2 μl water was added to the second aliquot. The samples were incubated at 37°C for 1 hr and bromophenolblue was added to a final concentration of 0.008% (w/v). .
Protein samples were loaded on a 13% SDS-polyacrylamide gel (Laemmli, 1970) (Bio-Rad mini protean system). After electrophoresis the proteins were transferred to a PVDF membrane (Millipore) by electroblotting. For immunodetection the membranes were incubated with 1:1000 diluted 9E10 monoclonal antibody (Munro and Pelham, 1986), followed by a 1:5000 diluted rat-anti-mouse alkaline phosphatase conjugate (Jackson Immuno Research). The blots were stained in 0.1 M ethanolamine-HCl pH 9.6, supplemented with 4 mM MgCl2, 5-bromo-4-chloro-3-indolyl phosphate (0.06 mg/ml) and nitro blue tetrazolium (0.1 mg/ml). The relative molecular weights of the proteins were estimated with pre-stained low range molecular weight markers (Bio-Rad).

Potato transformation and protein analysis.
The scFv48 expression cassettes cloned in the transient expression vector pTR2 were isolated as *PacI-AscI* fragments and transferred to the binary vector pBINPLUS as described by (van Engelen *et al.*, 1995). The resulting plasmids were introduced into *Agrobacterium tumefaciens* strain AGL0 (Lazo *et al.*, 1991). This strain was used for the transformation of internodal stem sections of *Solanum tuberosum*, dihaploid genotype 6487-9 (2n=2x=24), as described by (Flipse *et al.*, 1994). Genotype 6487-9 resulted from a crossing between genotype 1024-2 (Kuipers *et al.*, 1995) and genotype 91-6222-40, a self-incompatible Gg-clone selected in 1992 from the material of (Olsder and Hermsen, 1976). Kanamycin resistant transformants were regenerated and total soluble proteins were extracted by grinding roots essentially as described by (van Engelen *et al.*, 1994). The proteins were analyzed by loading 30 μg total soluble protein homogenate on an SDS-polyacrylamide gel followed by electroblotting and immundetection as described.

Results

Subcellular location of the scFv antibodies in plant cells.
To study possible 'mistranslocation' of scFv-CK to the ER we used N-linked glycosylation as a biochemical marker. A mutant, $scFv^{glyc}$-CK (Fig. 1), having a consensus glycosylation site (Asn-Gly-Ser) in the linker peptide was engineered. Both this construct and the construct having the original Ser-Gly-Ser peptide coding sequence in the linker ($scFv^{\Delta glyc}$-CK, Fig. 1) were expressed transiently in tobacco protoplasts. As controls we took the $scFv^{glyc}$-SK and $scFv^{\Delta glyc}$-SK constructs which carry both the ER translocation signal sequence and an ER retention signal KDEL (Fig. 1). Glycosylation was determined by the relative migration of these proteins on western blot (Fig. 2). $ScFv^{glyc}$-SK expression resulted in proteins migrating at 32, 33 and 67 kDa (Fig. 2, lane 1). The 67 kDa protein is thought to be an scFv dimer due to disulfide bridge formation caused by the cysteine present in the linker peptide (Fig. 1) (Schouten *et al.*, 1996). Due to the presence of β-mercaptoethanol in the reaction buffer this dimer was absent after glycosidase F treatment. When the transfected protoplasts were incubated in the presence of the glycosylation inhibitor tunicamycin or after glycosidase F treatment of the total protein homogenate, the 33 kDa band disappeared and the 67 kDa

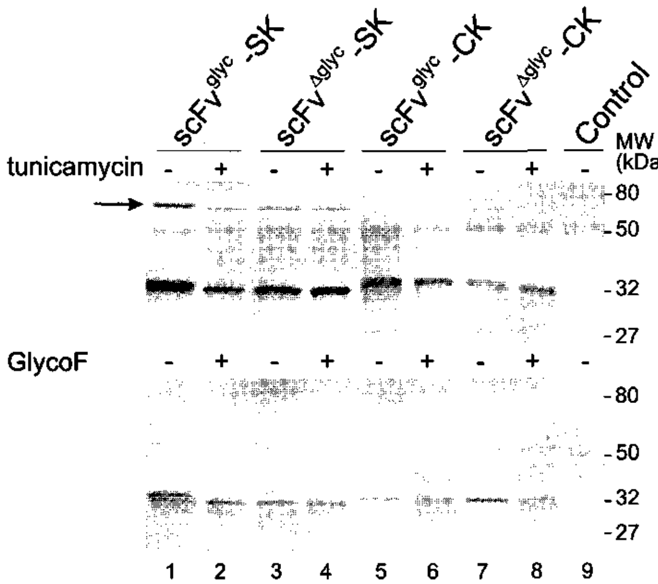

Fig. 2. Determining the glycosylation state of scFv^{glyc}-SK and scFv^{glyc}-CK proteins. Top panel: Western blot of total protein from tobacco protoplasts, transfected with the vectors containing the scFv^{glyc}-SK, scFv$^{\Delta glyc}$-SK, scFv^{glyc}-CK and scFv$^{\Delta glyc}$-CK gene cassettes, were incubated in the absence (-) or presence (+) of tunicamycin and analyzed on westernblot. Arrow indicates the 67/65 kDa protein bands. Bottom panel: Western blot of total protein from protoplasts, transfected with the vectors containing the scFv^{glyc}-SK, scFv$^{\Delta glyc}$-SK, scFv^{glyc}-CK and scFv$^{\Delta glyc}$-CK gene cassettes, which had been incubated in the absence of tunicamycin and were either mock treated (-) or treated (+) with the endoglycosidase glycoF. As a negative control (Control) a vector without scFv gene cassette was used.

band slightly shifted to 65 kDa (Fig. 2, lane 2). Expression of scFv$^{\Delta glyc}$-SK resulted in 32 and 65 kDa bands which were unaffected by glycosidase F treatment of the total protein homogenate or tunicamycin treatment of the transfected protoplasts (Fig. 2, lanes 3 and 4). This indicates that the 33 kDa and 67 kDa proteins are glycosylated forms of the 32 kDa scFv monomer and 65 kDa scFv dimer. The band intensities of the 33 and 32 kDa bands indicate that approximately 70% of the scFv^{glyc}-SK protein became glycosylated. Because of a three amino acid extension at the N-terminus the apparent molecular weight of the expressed scFv^{glyc}-CK and scFv$^{\Delta glyc}$-CK proteins is slightly higher than the scFv$^{\Delta glyc}$-SK and unglycosylated scFv^{glyc}-SK proteins and was estimated at 33kDa (Fig. 2, lanes 5 and 7). For both scFv^{glyc}-CK and scFv$^{\Delta glyc}$-CK the mobility was unaffected by glycosidase F treatment of the total protein homogenate or tunicamycin treatment of the transfected protoplasts (Fig. 2, lanes 6 and 8). Therefore, we conclude that the scFv^{glyc}-CK protein is not glycosylated, indicating that the scFv-CK antibody, in contrast to the scFv-SK antibody, is not translocated into the ER.

To obtain 'visual' proof for the presence of the scFv-CK antibodies in the cytosol, we examined leaf sections of transgenic plants expressing the anti-cutinase scFv-CK and scFv-SK antibodies with immunoelectron microscopy (Fig. 3). We had obtained transgenic plants with maximum expression levels of 0.2% and 1% of total soluble protein for scFv-CK and

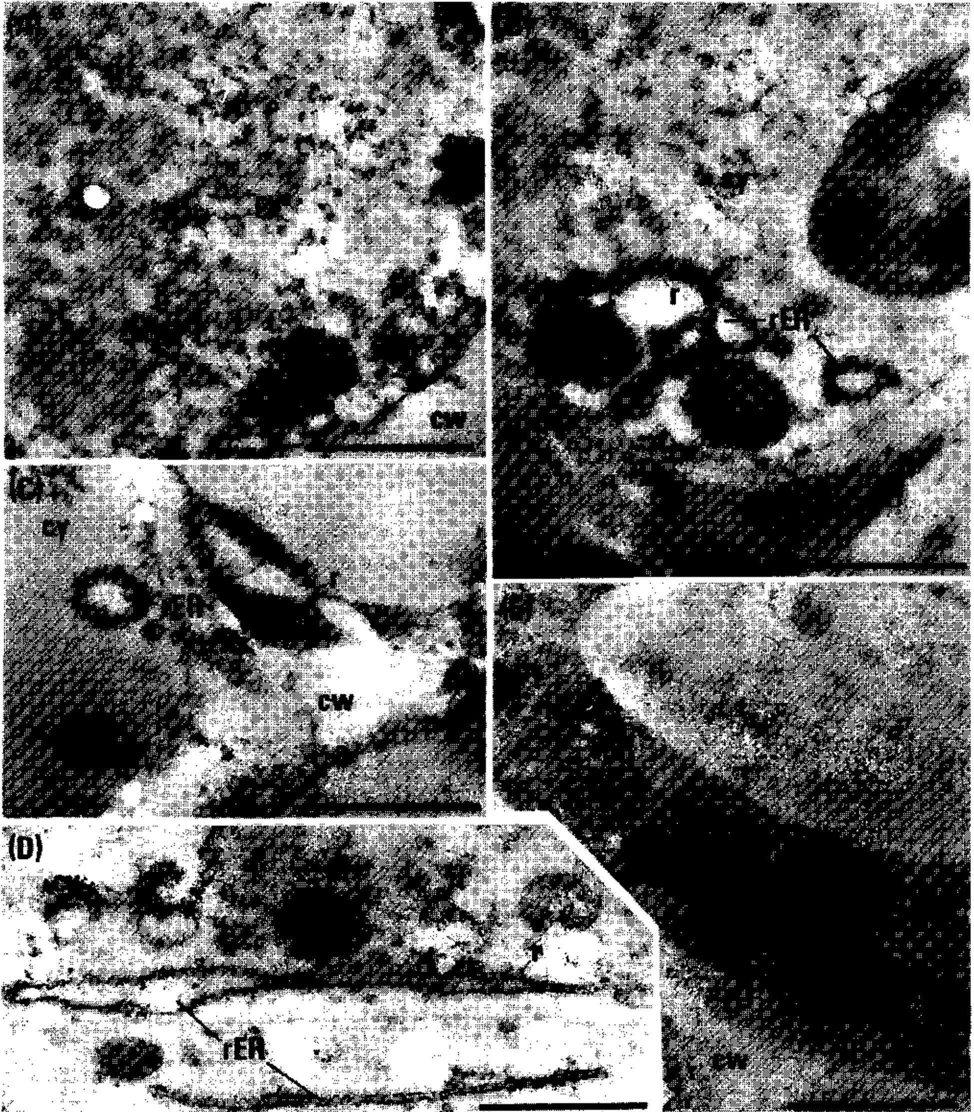

Fig. 3. Localization of scFv-CK and scFv-SK in transgenic tobacco by immunogold labeling and transmission electron microscopy. Leaf sections from plants expressing scFv-CK at a level of 0.2% (A and B) and scFv-SK at a level of 0.2% (C) and 1% (D) of total soluble protein. E: Leaf sections from untransformed plants. chl, chloroplast; cw, cell wall; cy, cytoplasm; rER, rough endoplasmic reticulum; r, ribosome. Bars = 1μm.

scFv-SK, respectively (Schouten *et al.*, 1996). Leaf sections were prepared from transgenic plants expressing the scFv-CK at a level of 0.2% and the scFv-SK at the levels of 0.2% and 1% of total soluble protein. The ultrathin sections of leaves expressing the scFv-CK showed gold particles evenly distributed in the cytosol (Fig. 3, A and B), indicating the presence of scFvs in that compartment. No gold particles were found in other subcellular compartments. The sections of the leaves expressing the ER-targeted scFv-SK showed gold particles localized in the membranous elements coated with electron dense ribosomes, the rough ER (rER) (Fig. 3, C and D). The number of gold particles correlated with the scFv-SK expression level. In several plant cells the structure of some of the rER was not elongated but vacuolated as has been described (Rodríguez-García *et al.*, 1995). No significant labeling was found in untransformed leaf sections (Fig. 3E). We therefore conclude that, as intended, the scFv-SK and scFv-CK were located in the ER and the cytosol, respectively.

Expression of different scFv antibodies in the cytosol of tobacco protoplasts.

To determine if the C-terminal KDEL extension could also improve cytosolic expression levels of other scFv antibodies the variable light (V_L) and heavy (V_H) domains of four other antibodies were amplified by PCR and coupled by a synthetic linker sequence in a 5'-V_L-linker-V_H-3' orientation. These antibodies were MGR48, MGR49 and MGR59, all reacting with β-1,4-endoglucanase, and anti-GUS, reacting with β-glucuronidase. The linker sequences coded for 202' (Pantoliano *et al.*, 1991) and $(Gly_4Ser)_3$ peptides or derivatives (Table 1). The scFv sequences were directly preceded by the ATG translational start codon and fused in frame with the c-myc tag coding sequence, enabling detection ('C' constructs). The 'CK' constructs carried the additional KDEL coding sequence at the 3'-end.

Table 1. Amino acid sequences of the linker peptides present in the various scFv antibodies.

Single-chain	Linker peptide
ScFv48	-LEGKSSGSGSESKLES-
ScFv49	$-(GGGGS)_3$
ScFv59	$-G_2S-(GGGGS)_3$
ScFvGUS	-REGKSSGSGSESKLES-

These scFv constructs were transiently expressed in tobacco protoplasts. The 21C5 anti-cutinase scFv-C and scFv-CK constructs were used as reference. Western blot analysis showed that cytosolic expression of the $scFv_{48}$ and $scFv_{59}$ constructs was improved when the KDEL tetrapeptide was present (Fig. 4). The estimated expression levels of $scFv_{48}$-C and $scFv_{48}$-CK were 0.02% and 0.06% of total soluble protein, respectively (Fig. 4, lane 1 and 2). The estimated expression levels of $scFv_{59}$-C and $scFv_{59}$-CK were 0.02% and 0.1% of total soluble protein, respectively (Fig. 4, lane 3 and 4). Poor expression was found with $scFv_{49}$ for both the C and CK versions. Transient expression of both $scFv_{GUS}$ versions gave no detectable expression (not shown). Apparently, the KDEL tetrapeptide is not capable of improving cytosolic expression of every scFv antibody.

Cytosolic expression of $scFv_{48}$ gene cassettes in transformed potato.

To determine whether the KDEL extension could improve scFv protein expression in the cytosol of another plant species we transformed C and CK gene constructs of $scFv_{48}$ to

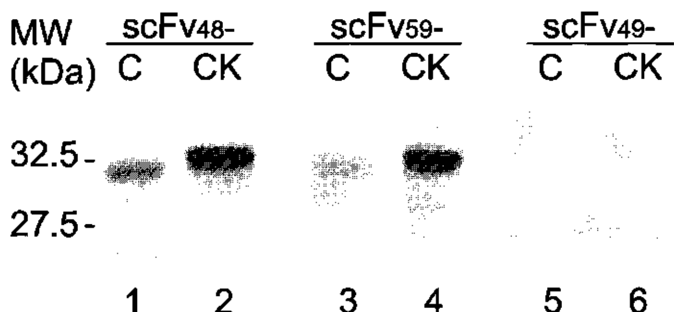

Fig. 4. Western blot analysis of a transient expression assay in tobacco protoplasts transformed with the scFv48, scFv59 and scFv49 constructs without (C) and with (CK) the tetrapeptide KDEL extension.

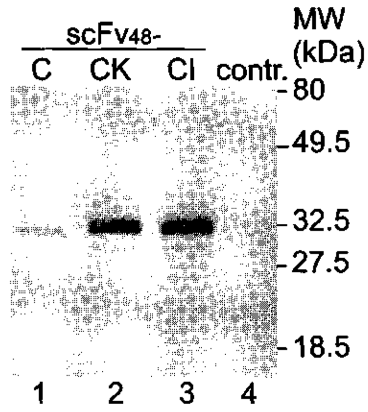

Fig. 5. Western blot analysis of transgenic potato plants expressing scFv48 without (C) and with (-CK) tetrapeptide KDEL extension or with tetrapeptide KDEI extension (CI).

potato by *Agrobacterium* mediated transformation. Independent kanamycin-resistant transformants were screened by immunoblotting of total protein extracts (Fig. 5). Only 20% of the scFv48-C transgenics screened showed expression, at an estimated level of 0.03% of total soluble protein (Fig. 5, lane 1). In contrast, 60% of the scFv48-CK transgenics screened showed an expression level estimated at 0.15-0.3% of total soluble protein (Fig. 5, lane 2). This demonstrates that the stabilizing effect of the tetrapeptide in cytosolic expression is not restricted to tobacco.

To study the influence of a modification in the KDEL extension on protein expression levels, an scFv48-CI gene construct, carrying the KDEI instead of KDEL coding sequence, was introduced into potato. This single amino acid substitution in the tetrapeptide is known to disrupt the retention signal and the protein is no longer retained in the ER (Denecke *et al.*, 1992). In 80% of the transgenics screened the scFv48-CI protein expression level was estimated at 0.3% of total protein (Fig. 5, lane 3), indicating that the isoleucine replacement did not result in a loss of the stabilizing effect.

Discussion.

The expression of antibodies in plants is a promising approach for obtaining pathogen resistance or altering metabolic pathways. Success depends on proper targeting to the desired subcellular compartment combined with proper folding and sufficient stability in order to obtain functional antibodies. The recent successes (Owen *et al.*, 1992; Tavladoraki *et al.*, 1993; Artsaenko *et al.*, 1995) suggest that scFv antibodies, provided with the proper translocation signals, have these abilities and can even be expressed in the reducing environment of the cytosol. However, these scFv antibodies were never properly localized and cytosolic scFv expression in plants was mostly low or absent. We demonstrated previously that the expression level of an anti-cutinase scFv antibody targeted to the cytosol was significantly enhanced when carrying the KDEL sequence C-terminally (scFv-CK) (Schouten *et al.*, 1996). Stabilizing scFv antibodies in the plant cytosol by adding this four amino acid sequence, known as ER retention signal (Denecke *et al.*, 1992), would open new possibilities, but the actual subcellular location was uncertain. Noteworthy in this respect is that for a cytosolic scFv antibody in HeLa cells mistranslocation to the ER was suggested (Jiang *et al.*, 1995). Furthermore, alternative translocation pathways to the ER of proteins lacking a signal sequence have been described (Muesch *et al.*, 1990; Rubartelli *et al.*, 1990 and 1992).

In this paper we located unambiguously the scFv-CK in the cytosol, excluding possible mistranslocation or alternative translocation events to the ER. This was proven using N-linked glycosylation as a biochemical marker and immunoelectron microscopy. N-linked glycosylation is a post-translational modification of proteins and confined to the ER (Czichi and Lennarz, 1977). Transient expression assays in tobacco protoplasts of the positive control, an scFv with ER translocation signal and KDEL extension carrying the glycosylation signal ($scFv^{glyc}$-SK), clearly demonstrated that glycosylation occurred, indicating that this scFv antibody had been translocated into the ER. However, only 70% of the $scFv^{glyc}$-SK became glycosylated. This could have been caused by the fact that the ER translocation signal was inefficient, and the $scFv^{glyc}$-SK was only partially translocated into the ER, or that N-linked glycosylation in the ER was incomplete. The first reason is most unlikely since immunoelectron microscopy showed no scFv-SK outside the ER of the plant cells. Incomplete glycosylation in the ER is more likely. Competition between protein folding and N-linked glycosylation has been described extensively (Allen *et al.*, 1995; Chen *et al.*, 1995; Holst *et al.*, 1996) and, apart from the X as a determinant in glycosylation efficiency (Shakin-Eshleman *et al.*, 1996), not all the Asn-X-Ser/Thr sequons in a protein become glycosylated (Gavel and Von-Heijne, 1990). Since the V_L domain can fold independently into a stable domain (Bergman and Kuehl, 1979; Freund *et al.*, 1996) the nascent V_L domain may rapidly begin with the formation of the proper domain structure the moment it is translocated into the ER, making it difficult for glycosyl transferase to attain the glycosylation signal in the linker peptide.

As reported before, the anti-cutinase scFv designed to be located in the cytosol could only be detected when the tetrapeptide KDEL was added C-terminally (Schouten *et al.*, 1996). We postulated that stabilization may have been caused by mistranslocation into the ER. In this case, all the scFv-CK antibody present would be located in the ER. However, the results showed that $scFv^{glyc}$-CK was not glycosylated, demonstrating that no mistranslocation into the ER had occurred. The actual subcellular location was confirmed by immunoelectron microscopy of tobacco leaf sections. The scFv-SK was detected in the ER and the scFv-CK

in the cytosol. No labeling was found in any other subcellular compartment. When two plants with similar expression levels are analyzed the scFv-CK antibody obviously becomes more diluted, since the cytosol, compared to the ER, is rather large in size. However, the immunodetection on the untransformed control plant showed no labeling. It can therefore be concluded that the four amino acid extension KDEL enhances the anti-cutinase scFv antibody expression levels in the cytosol.

Other scFv antibodies were constructed to investigate a broad applicability of the KDEL extension for stabilizing scFv antibodies expressed in the cytosol. Expression levels were not improved for one anti-β-1,4-endoglucanase scFv ($scFv_{49}$-CK) and the anti-β-glucuronidase scFv ($scFv_{GUS}$-CK). However, the other two anti-β-1,4-endoglucanase scFvs ($scFv_{48}$-CK and $scFv_{59}$-CK) showed a significantly enhanced protein expression level. This was found in both transient expression assays and stable transformants. As was already demonstrated previously (Schouten *et al.*, 1996) for cytosolic scFvs, the protein expression levels in transient expression assays with tobacco protoplasts are positively correlated with the levels obtained in transformed plants, even if this is a different species. Furthermore, the $scFv_{48}$-CK protein expression level in the transgenic plants, reaching 0.3% of total soluble protein, is very similar to what was found for the anti-cutinase scFv-CK (Schouten *et al.*, 1996).

How this four amino acid extension is capable of stabilizing the scFv antibody still remains elusive. An interaction with the transmembrane KDEL receptor is unlikely since the binding site of the receptor is exclusively located at the lumenal side of the ER membrane (Singh *et al.*, 1993). In addition, cytosolic scFv expression could also be improved when the tetrapeptide KDEI was added ($scFv_{48}$-CI). It was demonstrated before that the substitution of leucine into isoleucine disrupted the tetrapeptide to act as an ER retention signal (Denecke *et al.*, 1992). It may therefore well be that C-terminal protein degradation is prevented or that the tetrapeptide sterically protects a part of the scFv antibody susceptible for proteolysis.

Although different linker peptides were used to connect the variable domains of the different scFvs this seemed not to be crucial. The $scFv_{48}$, $scFv_{GUS}$ and the 21C5 scFv-CK (Schouten *et al.*, 1996) and $scFv^{\Delta glyc}$-CK all carried almost identical linker peptides. Yet, only the protein expression of the $scFv_{GUS}$ was not improved when the KDEL extension was added. Furthermore, the expression protein level of $scFv_{59}$-CK was considerably improved when compared to $scFv_{59}$-C. These scFvs both carried the $Gly_2Ser(Gly_4Ser)_3$ linker. Susceptibilty to proteolytic degradation may therefore depend on the amino acid sequence of the variable heavy and light domains. Since scFv antibodies lack the heavy and light chain constant domains some residues at the former variable and constant domain interface become solvent exposed. Depending on the amino acid sequence in this region proper folding and subsequent overall stability may be affected, thus increasing susceptibility to proteolytic degradation. Noteworthy in this respect is the recent report in which in COS-1 cells the cytosolic stability of an scFv antibody was greatly improved when the entire C_κ domain was added C-terminally (Mhashilkar *et al.*, 1995). Considering the negative results with $scFv_{49}$-CK and $scFv_{GUS}$-CK, apparently not all possible proteolytic sensitive sites are protected by the tetrapeptide extension.

As demonstrated with the cytosolic scFv expression in potato, the positive effect of the KDEL extension on the expression levels does not seem to depend on the choice of the plant species. The processes involved in stabilizing scFv antibodies in the cytosol of different species may be similar and is therefore not a factor determining the success of stable scFv expression.

It can be concluded that the tetrapeptide KDEL or KDEI can have a beneficial effect on the cytosolic expression levels of scFv antibodies in plants. Addition of this four amino acid extension may be a simple and effective solution for cytosolic scFv antibody expression and therefore is worth to try with scFvs which show great promise with respect to their binding affinity but can not be expressed at a sufficient level in the plant cytosol.

Acknowledgements:
The authors wish to thank G.A.M. (Ineke) de Jong, A.W.M. (Tanja) Borst-Vrenssen and J.W. Molthoff for technical assistance and Professor E. Jacobsen for providing *Solanum tuberosum* genotype 6487-9. This work was financially supported by grants from the Netherlands Technology Foundation (STW), coordinated through the Foundation for Life Sciences (SLW), and the European Commission (BIO2-CT92-0239; FAIR1-CT95-0905).

CHAPTER 5

Disulfide bridge formation within and between single-chain Fv antibodies in the reducing ectopic environment of the plant cytosol

Abstract

Disulfide bridge formation in the reducing environment of the cytosol is considered a rare event and is mostly linked to inactivation of protein activity. In this report the *in vivo* redox state of an scFv antibody fragment in the plant cytosol was investigated. The functionality of antibodies, which are normally secreted via the oxidizing environment of the ER, depends on the formation of disulfide bridges. We demonstrate that an scFv can form intramolecular disulfide bridges and is functionally expressed in the cytosol of stably transformed plants. In addition, the formation of intermolecular disulfide bridges through a cysteine present in the linker peptide was observed. However, transient expression in tobacco protoplasts resulted in an scFv antibody lacking disulfide bridges which had a substantially reduced affinity for the antigen. This indicates that functionality rather than stability is determined by the presence of disulfide bridges in the expressed scFv antibody in plants. The controversial role of disulfide bond formation in the cytosol is discussed.

Introduction

Disulfide bond formation and disulfide rearrangements are reversible processes, and are kinetically and thermodynamically affected by the redox state of the environment (Ziegler, 1985; Gilbert, 1990; Hwang *et al.*, 1992). In eukaryotic cells, subcellular compartmentation plays an important role in the regulation of these reversible thiol-disulfide exchange reactions. Glutathione is the most abundant nonprotein thiol in eukaryotic cells and the preferential transport of the disulfide form (GSSG) compared to the reduced form (GSH) into the ER lumen is thought to be responsible in maintaining redox compartmentation between the ER and cytosol. For mammalian cells, it has been shown that the ratio of reduced glutathione to the disulfide form (GSH/GSSG) ratio ranged from 1:1 to 3:1, whereas the overall cellular GSH/GSSG ratio ranged from 30:1 to 100:1 (Hwang *et al.*, 1992). *In vitro* studies have shown that the redox environment of the ER corresponds with the optimum for refolding of disulfide-bonded proteins (Hwang *et al.*, 1992). In the ER, disulfide formation and rearrangements are further enhanced by protein disulfide isomerase (PDI) (Gething and Sambrook, 1992).

As soon as the N-terminal part of a nascent polypeptide chain enters the oxidizing environment of the ER, folding starts with the aid of a full array of molecular chaperones and folding catalysts. Failure of disulphide formation has an adverse effect on various processes ranging from protein folding, oligomerization, intracellular transport to secretion (Alberini *et al.*, 1990; Braakman *et al.*, 1992; Tatu, *et al.*, 1993; Chanat *et al.*, 1993; Jämsä *et al.*, 1994; de Vries *et al.*, 1995). In extracellular proteins, containing cysteine residues, there is rarely more than one free thiol group (Thornton *et al.*, 1981). Free thiols are considered extremely reactive in the oxidizing extracellular environment and may lead to disastrous polymerization or make folding more complex.

The general idea is that the typical glutathione redox state of the cytosol in eukaryotic cells does not favour the formation of protein disulfide bonds (Thornton, 1981; Gilbert *et al.*, 1990). Also in prokaryotic organisms, intracellular disulfide bond formation seems to be hindered by the redox potential. Many recombinant proteins with disulfide bonds cannot fold properly in *E. coli*, where the intracellular GSH-GSSG redox state ranges from 50:1 to 200:1

(Hwang *et al.*, 1992). On the other hand, it has been suggested that the redox state of the cytosol in eukaryotes is not constant and may change in response to e.g. physiological and metabolic stimuli. Also specialized intracellular compartmentation may provide localized environments within the cell where the thiol-disulfide redox state may be very different than that of the bulk cytoplasm. However, apart from the comparison of the redox state of the ER with the intracellular environment as a whole, techniques to monitor changes or subtle subcellular redox compartmentation are nonexistent. Nevertheless, various lines of evidence indicate that redox modulation for some specific cytosolic proteins is an important regulatory mechanism in eukaryotic organisms (Hérouart *et al.*, 1993; Parks *et al.*, 1997). *In vitro* studies have demonstrated that various cytosolic enzymes are reversibly activated or inactivated upon incubation with disulfides or thiols (Abate *et al.*, 1990; Staal *et al.*, 1990). Disulfide bridges are often associated with an inactivated protein (Ziegler, 1985; Anderson *et al.*, 1995). These observations are consistent with metabolic changes in cell cultures, perfused organs and whole animals induced by manipulating the cellular thiol to disulfide balance by applying reducing agents (Rennenberg and Filner, 1982; Lodish and Kong, 1993; Tatu *et al.*, 1993; Garaci *et al.*, 1997). Although the biological significance of reversible thiol-disulfide exchange reactions for the cytosol remains controversial, in chloroplasts there is little question about the role of redox modulation in regulating the activity of key enzymes (Cséke and Buchanan, 1986; Gilbert, 1990). Also for nuclei evidence has been obtained that redox modulation influences the activity of transcription factors (Gilbert, 1990).
In this report we compared the redox state of an scFv antibody located in the ER and cytosol of tobacco (*Nicotiana tabacum*). We establish that in transgenic plants intramolecular disulfide bridges are present in both the cytosolic scFv and the scFv located in the ER. The cytosolic scFvs have binding properties which are similar to the scFv located in the ER. Furthermore, the scFvs present in the cytosol are, like the scFv in the ER, capable to form intermolecular disulfides through a cysteine present in the linker petide. Transient expression of the scFv in the ER of tobacco protoplasts also shows intramolecular and intermolecular disulfide bridges. However, no disulfide bridges are present when the scFv gene is expressed transiently in the cytosol. Although the expression levels are comparable to transgenic plants the binding properties of this cytosolic scFv are very poor. The relationships between transformation system, subcellular location, redox potential and disulfide bridge formation of heterologous proteins in the plant cytosol and the possible consequences are discussed.

Materials and methods

Cell lines, strains and cloning vectors.
A scFv directed against a fungal cutinase was derived from immunoglobulin cDNA of the hybridoma cell line 21C5 (Schouten et al., 1996). Cloning procedures were according to Sambrook et al., (1989) using the *Escherichia coli* strains DH5α ,TG1 and 190 and the scFv cloning vectors pNEM5 and pNEM5K (Schouten et al., 1996) For construction of the various 21C5 anti-cutinase scFv genes the pNEM-scFv-K (Schouten *et al.*,1996) was used.
Transgenic tobacco plants expressing scFv-SK and scFv-CK (Schouten *et al.*, 1996, 1997) were used for analysis. As a control we used untransformed tobacco (*N. tabacum* cv. Samsun NN) or a transgenic tobacco plant which was transformed with the empty transformation vector pCPO33T (Schouten *et al.*, 1996).

For transient expression assays pRAP-scFv-SK, pRAP-scFv-CK (Schouten *et al.*,1997) and, as a control, the empty transient expression vector pUCAP35S (van Engelen *et al.*, 1995) were used.

ScFv modifications.
In order to replace the Cys in the linker of the anti-cutinase scFv 21C5 by Ser, the *XhoI/PvuII* fragment in pNEM-scFv was replaced by a suitable adapter (5'-TCGAGTCTGAGGTCCAG-3' and 5'-CTGGACCTCAGAC-3') resulting in the pNEM-scFv^{ser} (Fig. 1). The scFv constructs were cloned as *SalI/NotI* fragments from pNEM-scFv^{ser} into pRAP-scFv-SK, creating and pNEM-scFv^{ser}-SK. All modifications were checked by sequencing (Sanger et al., 1977).

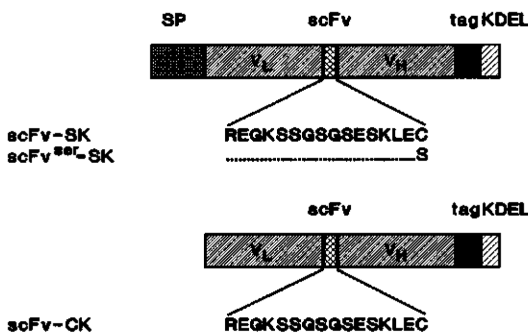

Fig. 1. Diagram of the scFv-SK and scFv^{ser}-SK constructs. In the scFv^{ser}-SK construct the cysteine present in the linker peptide was replaced by serine. The N-terminal signal peptide (SP), the single chain antibody construct (scFv) with the variable the variable light (V_L) and heavy (V_H) chain domain connected by the linker peptide, the C-terminal c-myc tag (tag) followed by the KDEL peptide sequence are indicated.

Preparation of protoplasts and transient gene expression.
Protolasts were obtained from transformed tobacco plants expressing scFv antibodies in the ER and cytosol at a level of 1% and 0.2 % of total soluble protein (Schouten *et al.*, 1996) by the method described by (Denecke *et al.*, 1989). As a control protoplasts were isolated from untransformed tobacco leaves (*N. tabacum* cv. Samsun NN)
For transient expression assays the same procedure was used to obtain protoplasts from tobacco leaves (*N. tabacum* cv. Samsun NN). Then plasmid DNA was introduced into the protoplasts according to the polyethylene glycol procedure as described by (Denecke *et al.*, 1989).

Analysis of intrachain disulfide bonds.
The protoplasts from stable transformants or from a transient expression assay, expressing both scFv-SK and scFv-CK proteins, were separated from the incubation medium and lysed by adding an equal volume of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM Pefabloc, 80 mM N-ethylmaleimide (NEM) and subsequent vortexing. The suspension was kept at 0°C for 5' and at room temperature for 10'. The chloroplasts were pelleted by centrifugation and to the the supernatant was split into two portions. The first portion received one forth volume

4×SDS-PAGE sample buffer, containing 244 mM Tris-HCl, pH 6.8, 8% (w/v) SDS, 50% (w/v) glycerol, and the second portion received 4×SDS-PAGE sample buffer and dithiothreitol (DTT) to a final concentration of 55 mM. Both samples were boiled for 5'. To the DTT containing portion NEM was added to a final concentration of 200 mM and incubated 15' at room temperature. The proteins were pelleted by ethanol precipitation and subsequent centrifugation. After drying the pellet was resuspended in 1×SDS-PAGE sample buffer with 0.008% (w/v) bromophenolblue. Protein samples were loaded on a 13% SDS-polyacrylamide gel (Leammli, 1970) (Bio-Rad mini protean system). After electrophoresis the proteins were transferred to a PVDF membrane (Millipore) by electroblotting. For immunodetection the membranes were incubated with 1:1000 diluted 9E10 monoclonal antibody (Munro and Pelham, 1986), followed by a 1:5000 diluted rat-anti-mouse alkaline phosphatase conjugate (Jackson Immuno Research). The blots were stained in 0.1 M ethanolamine-HCl pH 9.6, supplemented with 4 mM MgCl2, 5-bromo-4-chloro-3-indolyl phosphate (0.06 mg/ml) and nitro blue tetrazolium (0.1 mg/ml). The relative molecular weights of the proteins were estimated with pre-stained low range molecular weight markers (Bio-Rad).

Analysis of interchain disulphide bonds.
Protoplasts transiently expressing scFv-SK and $scFv^{ser}$-SK were separated from the incubation medium. The protoplasts were separated from the incubation medium and lysed by adding an equal volume of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 1 mM Pefabloc SC (Boehringer), and subsequent vortexing. The chloroplasts were pelleted by centrifugation at 13000×g for 2'. For western analysis one third volume 4×SDS-PAGE sample buffer was added to an aliquot of the supernatant, equaling 3×10^4 protoplasts. DTT and bromophenolblue were added to final concentrations of 40 mM and 0.008% (w/v), respectively, and the samples were boiled at 100°C for 5 minutes. For non-reducing gel electrophoresis DTT was omitted during sample preparation. The protein samples were analyzed by SDS-polyacrylamide gel followed by electroblotting and immunodetection as described.

Affinity of oxidized and reduced anti-cutinase scFv for its antigen.
The affinity of the oxidized and reduced scFv for cutinase was determined by both quantitative ELISA. To prevent disulfide bridge formation during sample preparation alkylated protein samples were prepared as followed. The proteins were extracted by grinding 0.2-0.4 g tobacco leaves in liquid nitrogen to a fine powder. The powder was transferred to an eppendorf tube and mixed 1:2 (w/v) with 10mM Tris-HCl, pH8.0, 5mM EDTA, 60mM NEM, 1mM Pefabloc SC (Boehringer) and subsequent vortexing. As a control extracts were prepared in the presence of 4% ethanol (the solvent of NEM) instead of NEM. The suspension was kept at 0°C for 5' and at room temperature for 10'. Insoluble material was removed by centrifugation at 13.000×g. To the supernatant 25% (v/v) 5×PBS, 0.5% (v/v) Tween, 5% (w/v) skimmed milk powder was added. Serial dilutions were made in 1×PBS-0.1% (v/v) Tween (PBST), 1% (w/v) skimmed milk powder, and loaded on an ELISA plate.
After a transient expression assay the protoplasts, transfected with pRAP-scFv-CK, pRAP-scFv-SK and pUCAP35S, were separated from the incubation medium and lysed by adding an equal volume of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 1 mM Pefabloc SC (Boehringer), and subsequent vortexing. The suspension was kept at 0°C for 5' and at room

temperature for 10' and the chloroplasts were pelleted by centrifugation at 13000×g for 2'. The supernatant was diluted with 5 volumes of PBST-1.25 % (w/v) skimmed milk powder and loaded on a 96-well ELISA plate which had been coated overnight with 2.5 μg/ml cutinase in 50 mM sodium carbonate, pH 9.6 (100 μl/well). After blocking for 30 minutes with 200 μl PBST-5% skimmed milk powder per well the plates were washed and 100 μl protein extract per well was added. The plate was incubated for 2 hours. After washing with PBST each well was incubated for another two hours with 100 μl anti c-myc tag antibody 9E10 (1 ng/μl) in PBST-1% skimmed milk powder. Then, after washing three times with PBST, the wells were incubated for 1 hour with alkaline phosphatase conjugated rat-anti-mouse antibody (Jackson Immuno Research), diluted 1:5000 in PBST-1% skimmed milk powder. Finally the wells were washed five times with PBST. 100 μl Substrate (0.75 mg/ml p-nitrophenylphosphate in 1M diethanolamine, pH 9.8) was added and the OD_{405} was monitored. All incubations were carried out at 37°C.

Results

Presence of disulphide bonds in the scFvs located in the ER and cytosol.

We previously generated transgenic tobacco plants expressing functional scFv-CK and scFv-SK antibodies which were located in the cytosol and ER, respectively (Schouten *et al.*, 1996, 1997). Therefore, the scFv-SK antibody contains a signal sequence, for translocation into the ER, combined with the ER retention signal KDEL (Pelham, 1989). The scFv-CK, located in the cytosol, lacks the ER translocation signal. All scFv antibodies carry the *c-myc* tag sequence for detection purposes.

To compare the *in vivo* redox state of the disulfide bridges in the cytosolic scFv-CK and ER located scFv-SK synthesized in the transgenics and in a transient expression assay we isolated protoplasts from transgenic tobacco leaves according to the procedure used for a transient expression assay. The protoplasts were cooled and immediately lysed in the presence of the sulphydryl alkylating agent N-ethylmaleimide (NEM), to block free thiols and prevent rearrangements of disulfide bonds. The scFv proteins were analyzed by SDS-PAGE followed by western blotting and immuno-detection using the anti-c-myc antibody, 9E10 (Fig. 2). The transgenic tobacco protoplasts showed under non-reducing conditions for both scFv-CK and scFv-SK a protein band at 32 kDa, the estimated molecular weight of the scFv antibodies, and at approximately 65 kDa (Fig. 2A, lanes 1 and 3). In the presence of the reducing agent DTT only a protein band at 33 kDa was detected (Fig. 2A, lanes 2 and 4). No protein bands were detected in protoplasts obtained from the plants transformed with the empty transformation vector pCPO33T (Fig. 2A, lane 5). Apparently, as reported before (Biocca *et al.*, 1995), the reduction of the two intramolecular disulfide bridges in an scFv antibody results in the shift in migration from 32 to 33 kDa. The 65 kDa band present in both the scFv-CK and scFv-SK is only present under oxidizing conditions (i.e. in the absence of DTT) and, as reported before (Schouten *et al.*, 1996), shows antigen binding capabilities. This suggests it to be an scFv dimer, formed through intermolecular disulfide brigde formation by the cysteine residue present in the linker peptide (Fig. 1). Thus in transgenic tobacco both the cytosolic scFv-CK and ER located scFv-SK are present in a oxidized state.

The protoplasts from the transient expression assay synthesizing scFv-SK also showed the 32 kDa and 65 kDa protein bands under non-reducing conditions and the 32.5 kDa band under reducing conditions (Fig 2B, lanes 3 and 4). Both under non-reducing and reducing

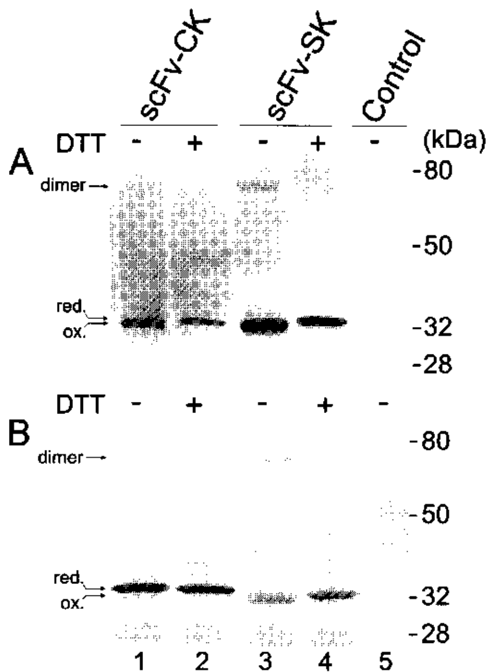

Fig. 2. Determining the redox state of cysteine residues present in scFv-SK and scFv-CK proteins expressed by stably transformed plants and in a transient expression assay. Panel A: Western blot of total protein from stably transformed tobacco plants expressing scFv-CK and scFv-SK, in the absence (-) or presence (+) of the reducing agent dithiothreitol (DTT). The scFv proteins were detected with the anti-c-myc antibody 9E10. The reduced (red.) and oxidized (ox.) scFv proteins and the presumed scFv dimer (dimer) are indicated. As control a tobacco plant stably transformed with the empty transformation vector pCPO33T was used (Control). Panel B: Western blot of total protein from protoplasts, transfected with the vectors containing the scFv-CK and scFv-SK gene cassettes, in the absence (-) or presence (+) of the reducing agent DTT. The scFv proteins were detected with the anti-c-myc antibody 9E10. The reduced (red.) and oxidized (ox.) scFv proteins and the presumed scFv dimer (dimer) are indicated. As a control a vector without scFv gene cassette was used (Control).

conditions the protoplasts from the transient expression assay synthesizing scFv-CK showed the 32.5 kDa (Fig 2B, lanes 1 and 2). Only a weak background was detected in the transient expression assay when the empty expression vector pUCAP35S was expressed (Fig. 2B, lane 5). Therefore, in contrast to scFv-SK, the transiently expressed scFv-CK lacks the intramolecular and intermolecular disulfide bridges and thus is present in a reduced state. This situation differs from the oxidized scFv-CK present in stable transformed plants.

Analysis of the scFv dimer formation.

To ascertain that the 65 kDa protein observed in the transgenic plants is formed through disulfide bridge formation using the cysteine present in the linker we modified the linker region in the scFv-SK gene (Fig. 1). The cysteine coding triplet (TGT) was changed into a serine coding triplet (TCT), creating the $scFv^{ser}$-SK gene. We anticipated that, if the cysteine residue in the linker peptide was involved in dimer formation, $scFv^{ser}$-SK expression would

not result in the formation of the disulfide band under non reducing conditions. We only modified the scFv-SK gene since we wanted to analyze the 65 kDa band through a transient expression assay. Since, as mentioned before, the scFv-CK synthesized in a transient expression assay does not form disulfide bridges a transient expression assay with a modified scFv-CK would be useless to obtain the required information.

The scFv-SK and scFv^{ser}-SK genes were both expressed transiently in tobacco protoplasts and the synthesized scFv proteins were analyzed by western blotting and immuno-detection, using the anti-c-myc antibody, 9E10 (Fig. 3). Under non-reducing conditions scFv-SK expression resulted in a weak 32 kDa and relatively strong 65 kDa protein band and the scFv^{ser}-SK expression resulted only in a 32 kDa protein band (Fig. 3, lanes 1 and 2). Under reducing conditions both scFv-SK and scFv^{ser}-SK expression resulted in 32 kDa protein bands (Fig. 3, lanes 4 and 5). Under both conditions, only a weak background was detected in the transient expression assay with the empty expression vector pUCAP35S (Fig. 3, lanes 3 and 6). The 65 kDa band is only formed under non-reducing conditions when the cysteine is present in the linker peptide, indicating that the 65 kDa protein represents a dimerized scFv.

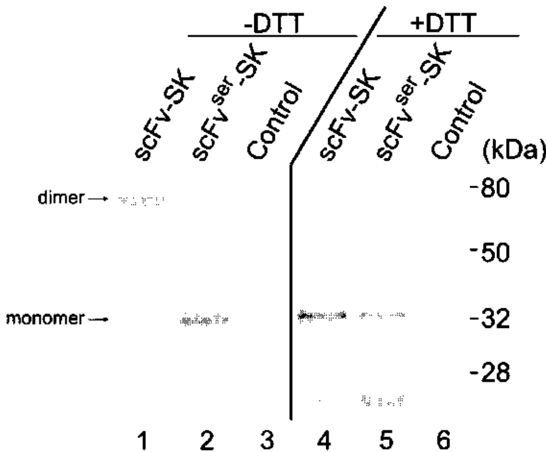

Fig. 3. Determining the role of the cysteine residue present in the linker peptide of scFv-SK in dimer formation. Western blot of total protein from protoplasts, transfected with the vectors containing the scFv-SK and scFv^{ser}-SK gene cassettes, in the absence (-DTT) or presence (+DTT) of the reducing agent dithiothreitol. The scFv proteins were detected with the anti-c-myc antibody 9E10. The scFv monomer and dimer proteins are indicated. As a control a vector without scFv gene cassette was used (Control).

Redox state influences binding properties of scFv-CK.

We are very interested if the redox state of the 21C5 anti-cutinase scFv affected binding to the antigen cutinase. To determine the affinity by ELISA, we first prepared samples from transgenic tobacco leaves expressing scFv-CK, scFv-SK and, as a control, untransformed tobacco (*N. tabacum* cv. Samsun NN) in the presence of NEM. To exclude possible negative effects on the affinity by NEM (Goto and Hamaguchi, 1979) we also prepared samples in the absence of NEM. The concentration of the scFv in these crude protein homogenates (Fig. 4A, insert) was calculated by comparison with different concentrations of purified Ec-scFv.

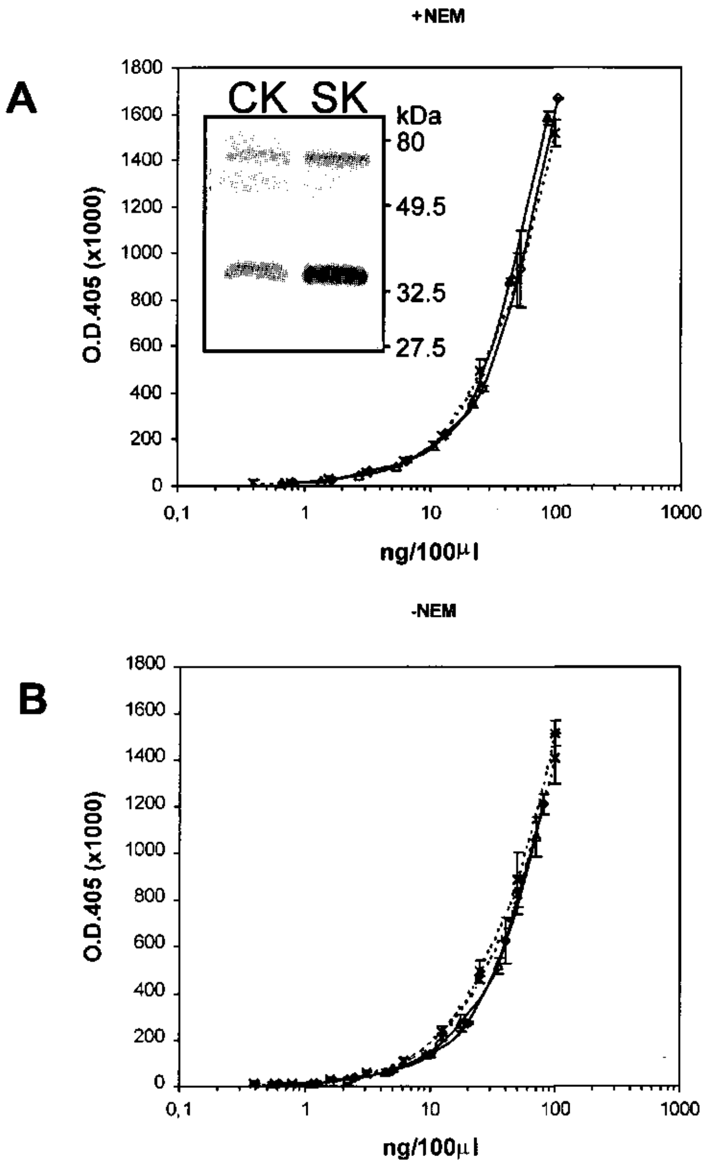

Fig. 4. Antigen-binding activity of scFv antibodies present in crude protein homogenates from leafs of stably transformed tobacco plants, assayed by ELISA using the anti-c-myc antibody. A western blot to detect scFv-CK and scFv-SK (Fig. 4A, insert) with the anti-c-myc antibody 9E10 was used for calculating scFv concentrations by comparison with different concentrations of purified scFv from *E.coli*. Serial dilutions of the scFv-CK (▵)and scFv-SK (◇) protein homogenates and purified scFv from *E.coli* (Ec-scFv) (×, -NEM; □, +NEM) were incubated in wells coated with 250 ng cutinase. Individual points represent mean values of duplicate trials with standard deviations (error bars). Protein samples were prepared in the presence (Panel A; +NEM) and in the absence (Panel B; -NEM) of the sulphydryl alkylating agent N-ethylmaleimide.

the 21C5 anti-cutinase scFv expressed in the periplasm of *E. coli* (Schouten *et al.*, 1996), on SDS-PAGE followed by western blotting and immuno-detection using the anti-c-myc antibody, 9E10.

Serial dilutions, in duplicate, of the scFv-SK and scFv-CK and untransformed tobacco samples were loaded on an ELISA plate coated with cutinase. As a reference duplicate serial dilutions of NEM treated purified Ec-scFv were included in the assay. Fig. 4A shows the comparison of the antigen-binding activity of scFv-CK, scFv-SK, both in the crude trangenic plant exctracts, and purified Ec-scFv. Apparently, the binding properties are very similar. The plant proteins in the crude homogenates did not affect the OD_{405} values, as was deteremined for the control samples, prepared from untransformed plants (not shown). Treatment with NEM does not affect the antigen binding properties of the anti-cutinase scFv since the samples prepared in the absence of NEM also show no significant difference in antigen-binding activity in the same experiment (Fig. 4B).

Next NEM treated samples were prepared from the protoplasts transfected with the scFv-CK and scFv-SK expression vectors and the empty vector pUCAP35S as described before. As shown the migration rate and the presence of the 65 kDa protein band indicates the redox state of the scFv antibody. Western analysis of the protoplasts showed for scFv-SK the 32 and 65 kDa band, indicating the oxidized nature, and for scFv-CK the 33 kDa protein band indicating the reduced nature (Fig. 5A). The concentration of the scFv in these crude protein homogenates was calculated at 8 ng/µl for both scFv-SK and scFv-CK by comparison with different concentrations of Ec-scFv on SDS-PAGE followed by western blotting and immuno-

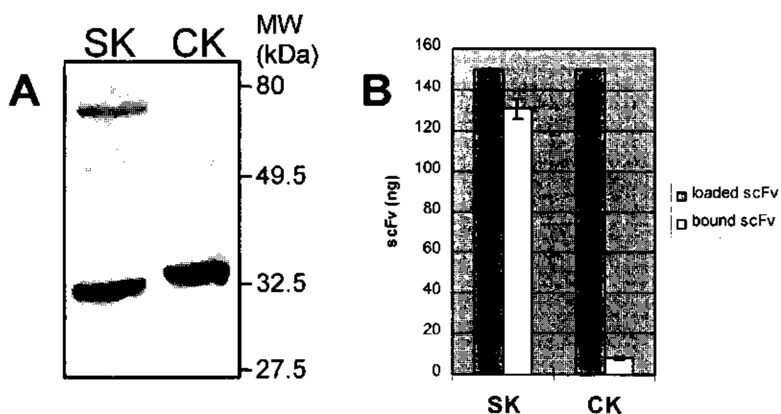

Fig. 5. Binding of transiently expressed scFv-SK and scFV-CK antibodies to cutinase. Panel A: Western blot of total protein homogenates prepared in the presence of NEM from protoplasts, transfected with the vectors containing the scFv-CK and scFv-SK gene cassettes in the absenceof the reducing agent DTT was used for calculating scFv concentrations by comparison with different concentrations of purified scFv from *E.coli*. The scFv proteins were detected with the anti-c-myc antibody (9E10). Panel B: Protein homogenates containing 150 ng of the scFv-CK and scFv-SK and serial dilutions of scFv purified from *E.coli* were incubated in wells coated with 250 ng cutinase in triplicate. The serial dilutions were used to calculate the amount of bound scFv-SK and scFV-CK to cutinase. The bars represent the mean values of bound scFv-SK and scFV-CK with standard deviations (error bars), corrected for the protoplast homogenate transfected with the epmty vector pUCAP35S. The antibodies were detected with the anti-c-myc antibody (9E10).

detection using the anti-c-myc antibody, 9E10. ELISA samples were prepared and an ELISA binding assay was performed in triplicate using 150 ng scFv-CK and scFv-SK. To calculate binding serial dilutions of NEM treated Ec-scFv was included, in duplicate, in the assay. Fig. 5 shows the comparison of the antigen-binding activity of scFv-CK and scFv-SK, both in the crude trangenic plant exctracts, and Ec-scFv. Since the antigen-binding activity of the Ec-scFv was similar to oxidized scFv-SK and scFv-CK we calculated from the Ec-scFv reference that the OD_{405} value correlated to a concentration of 130.8±5.1 ng scFv-SK per well, after correcting for the background caused by aspecific binding of plant proteins from the crude homogenate (Fig. 5B). The scFv-CK OD_{405} correlated to 8.0±0.8 ng per well. It can therefore be concluded that in comparison to the oxidized scFv-SK less than 10% of the scFv-CK remained bound to cutinase. The alkylation of the cysteines by NEM did not cause the reduction in binding strength of the reduced scFv-CK as was determined by sample preparation in the absence of NEM (not shown). This indicates that the reduced scFv-CK antibody has a considerable reduced binding strength when compared to the oxidized scFv-SK and scFv-CK. Apparently, the absence of intramolecular disulfide bridges has a considerable negative effect on the binding strength of the anti-cutinase scFv.

Discussion

The capability of an scFv antibody to form disulfide bridges in the cytosol is remarkable. Protein disulfide isomerase (PDI), a catalyst in the oxidative folding, is not present in the cytosol and the GSH/GSSG ratios of 30:1 to 100:1 (Hwang *et al.*, 1992) do not favor disulfide bridge formation. It was calculated that RNase A, which efficiently refold at a GSH/GSSG ratio of 2:1 in the presence of PDI, would not be able to refold at the cytosolic GSH/GSSG ratios, both in the presence and absence of PDI (Hwang *et al.*, 1992). This demonstrates that disulfide bridge formation in proteins is very much dependent on the GSH/GSSG ratio of the subcellular compartment. Cell-free translation assays in the presence of PDI and added bacterial chaperones showed that an scFv antibody fragment folds better at mildly reducing conditions, *i.e.* a GSH/GSSG ratio of 10:1 at 1.1 mM total glutathione, then at oxidizing conditions (Ryabova *et al.*, 1997). The bacterial chaperones did not promote the proper disulfide bridge formation and it was suggested that PDI merely served in disulfide rearrangements rather than in disulfide formation. Our results indicate that *in vivo* disulfide bridge formation in scFvs is possible at GSH/GSSG ratios found in the plant cytosol. Consequently, if these ratios correspond to the ratios found in the cytosol of cultured CRL-1606 hybridoma cells (Hwang *et al.*, 1992) the scFv antibody is capable of forming disulfide bridges at GSH/GSSG ratios of 30:1 or higher. However, it is possible that in plants this ratio deviates or that the GSH concentration is relatively low. The total GSH/GSSG ratio of the phloem exudates of *Cucurbita* (Alosi *et al.*, 1988) and *Ricinus* (Szerderkényi *et al.*, 1997) was calculated at 13:1 and 3:1, respectively. Since phloem exudates represents the cytosol of a plant cell this total GSH/GSSG ratio represents the cytosolic ratio. In addition, in pumpkin leaves low concentrations of total GSH were measured (Rennenberg and Filner, 1982). The concentration of GSH also has great influence on disulfide bridge formation in proteins (Gilbert, 1990)

The presence of certain eukaryotic cytosolic chaperones (Ruddon and Beddows, 1997) may facilitate the efficient folding of the V_L and V_H domains and prevent aggregation through erroneous disulfide bridge formation. Once folded into the native structure, the disulfide

bridges are inaccessible for reduced glutathion due to a tight surrounding structure (Glockshuber *et al.*, 1992; Goto and Hamaguchi, 1979). That folding is accurate is emphasized by the 202' linker derivative with the cysteine residue. Even this extra cysteine does not result in an abberant disulfide bridge formation and results in a partial but rather organized dimerization.
As demonstrated by the transient expression experiments, the plant cytosol can also be reducing enough to prevent disulfide bridge formation. This confirms that the redox state of the plant cytosol is not fixed and that redox modulation may play an important role in the physiology of the plant cell as suggested before. Why the cytosol of protoplasts is more reducing remains a question. Incubation of the protoplasts after transfection occurs in the dark and therefore the cytosol is presumed to be more oxidized when compared to illuminated protoplasts (Cséke and Buchanan, 1986). This should favor disulfide bridge formation. It may well be that the protoplasts, which are kept under non-natural conditions, protect themselves against an oxidative stress situation. The levels of GSH in foliar tissues has been shown to increase under various oxidative stress conditions (Alscher, 1989). Furthermore, extracellular added GSH was also shown to act as an activator of the transcription of genes encoding the cell wall hydroxyproline-rich glycoproteins and phenyl propanoid biosynthetic enzymes in cultured suspension cells or protoplasts of bean, soybean, and alfalfa (Wingate *et al.*, 1988; Choudhary *et al.*, 1990).
Our results also raise the question whether such oxidative stress reactions also apply for other eukaryotic cell types. In other words, is the GSH/GSSG ratio in the cytosol of cultured animal cells higher than the ratio in the cytosol of the tissue from which the cells are derived? If in all cases the scFv functionality is indeed a marker for the presence of disulfide bridges, the reported successes with respect to cytosolic expression of scFvs for introducing resistance against pathogens, like viruses (Tavladoraki *et al.*, 1993; Duan *et al.*, 1994; Jiang *et al.*, 1995; Mhasilkar *et al.*, 1995; Shaheen *et al.*, 1996), or modifying phenotypic traits (Owen *et al.*, 1992; Biocca *et* al., 1993 and 1994; Werge *et al.*, 1994; Fecker *et al.*, 1997) would imply the presence of disulfide bridges. However, Biocca *et al.* (1995a) reported that a functional cytosolic anti-p21^{ras} scFv, expressed in *Xenopus* oocytes, lacked disulfide bridges. To date, nothing is known about disulfide bridge formation in scFv antibodies in the cytosol of other eukaryotic cell types.
As the results show, scFv antibodies can also form intermolecular disulfide bridges in the plant cytosol. Maciejewski *et al.* (1995) reported the functional expression of an anti-HIV1 reverse transcriptase Fab fragment in the cytosol of lymphoid cell lines, leading to a blockade of virus replication. Although it was demonstrated that uninfected cells expressed unassembled Fab fragments, i.e. without intermolecular disulfide bridges, it may have been that assembly may have occured during infection since HIV infection strongly reduces the intracellular reduced glutathion concentration (Garaci *et al.*, 1997).
Our results also demonstrate that scFv antibodies without disulfide bridges can be found as a soluble protein in the plant cytosol and its stability does not seem to be affected. This is in contrast to heterologous scFv expression in *E. coli* (Glockshuber *et al.*, 1992) where the formation of the intramolecular disulfide bonds in both the variable heavy and light domains are considered crucial for stability. Apparently, next to the redox potential, the environment of the plant cytosol differs in other aspects from the *E. coli* cytosol. It was demonstrated by Freund *et al.* (1997) that *in vitro* an scFv antibody folds through an early and fast folding intermediate followed by a slow folding proces into the final structure. As mentioned, certain chaperones, not present in the cytosol of *E. coli*, may play an important role in stabilizing and

maintaining a folding intermediate of the scFv antibody. It may therefore be that the disulfide bridge formation required for the final folding steps to acquire the fully folded structure is prevented due to the reducing environment.

This report illustrates that it is impossible to predict a *priori* to what extent a given protein sulphydryl will remain reduced in the cytosol. This was also suggested by Piñeiro *et al.* (1994). Bacterial genes coding for the cytosolic enzymes phoshotransferase II (NPTII) and β-glucuronidase (GUS) were transiently expressed in the cytosol of tobacco protoplasts. Since the typical GSH/GSSG ratio in *Escherichia coli* ranges from 50:1 to 300:1 they reasoned that the GSH/GSSG ratio of 30:1 to 100:1 in the plant cytosol was not reducing enough to prevent disulfide bridge formation in these enzymes, resulting in a reduced stability. Thus, in cases disulfide bridges determine functionality and/or stability of a protein, the result of heterologous expression of the coding gene may be difficult to foresee.

Expression in plant cytosol of functional scFvs has proven to be feasible and therefore offers good perspective with respect to their use in intracellular immunization and immuno modulation approaches. However, the choice of the expression system should be made carefully and guarantee a redox state of the heterologously expressed protein which represents the situation in the natural tissue. Transient expression assays may therefore be only representative for protein expression levels and not for functionality in the cytosol.

CHAPTER 6

ScFv stability in the secretory pathway of plant cells: Influence of linker peptides, the scFv architecture, C_H1 residues and the addition of camel hinges and IgM C_H4 domains

Abstract

Single-chain antibody fragments (scFvs) are often poorly secreted by plants. This may be caused by the primary amino acid sequence of the Fv domains, the orientation of the V_L and V_H domains in the scFv construct, the choice of the linker peptide and the amino acids present at the C-terminal ends. It was investigated whether scFvs could be secreted more efficiently by modifying the elements necessary for constructing an scFv, without changing the Fv domains. Different linker peptides were compared. Amino acids potentially susceptible to degradation in one linker were replaced. ScFvs were constructed in V_L- V_H and V_H -V_L orientation, relative to the N- and C-terminus of the protein. Residual C_H1 fragments were removed. Although, some of these modifications did increase intracellular accumulation of partially degraded scFv, secretion was not improved. However, addition of an IgM C_H4 domain and camel long or short hinges, for the purpose of multimerizing scFv fragments, did improve both the intracellular accumulation and the secretion of non-degraded scFv fragments. Quality control mechanisms involved in proper folding and processing of antibody fragments in the secretory pathway are discussed.

Introduction

ScFv expression in the secretory pathway of plants is generally poor (Bruyns *et al.*, 1996; Fecker *et al.*, 1996; Whitelam and Cockburn, 1996), unless the scFv was retained in the endoplasmic reticulum (ER) using a C-terminal KDEL sequence (Artsaenko *et al.*, 1995; Schouten *et al.*, 1996 and 1997; Phillips *et al.*, 1997). Only Firek *et al.* (1994) obtained substantial secretion of an scFv antibody in tobacco leafs and Fiedler and Conrad (1995) reported expression of a secretory scFv in tobacco seeds when using a seed specific promoter.
So far, analysis of the expression pattern of chimeric scFv genes showed that the transcription levels obtained were in general normal, indicating that bottlenecks are predominated by (post-)translational processes.
Expression in mammalian cells showed that different scFvs were secreted at various rates and that secretion could be improved by changing specific amino acids (Jost *et al.*, 1994). Studies with scFv molecules, produced in *Escherichia coli*, have shown that the linker connecting the variable domains may have an impact on scFv stability. The number of amino acid residues (Pantaliano *et al.*, 1991; Whitlow *et al.*, 1993; Desplancq *et al.*, 1994) and the residues used (Glockshuber *et al.*, 1990; Yasui *et al.*, 1994) influence scFv folding, aggregation and its sensitivity to proteolytic degradation. Also, the scFv architecture (V_L-V_H versus V_H-V_L) has been shown to influence expression not only in *E. coli* (Anand *et al.*, 1991; Tsumoto *et al.*, 1994) but also in mammalian cells (Dorai *et al.*, 1994). Some scFvs often include residues from the constant domains for cloning purposes. The stability of such scFv-constructs might be affected by these amino acid sequences. Also, the addition of whole antibody domains, for the purpose of dimerizing scFvs and generating a $F(ab')_2$-like molecules, has been reported to stabilize scFvs *in vivo* (Hu *et al.*, 1996).
elements by introducing them into the model scFv 21C5, which is poorly secreted in transgenic tobacco (Schouten *et al.*, 1996). The 202' derived peptide linker (Bird *et al.*,

1988), present in the initial 21C5 scFv (see: Chapter 3), was modified as well as replaced by the frequently employed $(Gly_4Ser)_3$ linker (Huston *et al.*,1988). The scFv was constructed in both V_L-V_H and V_H-V_L architecture. Furthermore, the first five amino acids of the C_H1 domain, initially present in scFv 21C5, were deleted. Finally, to monitor the efficiency of multimerization and its influence on expression we added the camel long hinge or short hinge (Hamers-Casterman, *et al.*, 1993) and/or the IgM C_H4 domain (Smith and Morrison, 1994) to the scFv antibodies. Using transient assays in tobacco leaf protoplasts and stably transformed tobacco plants we monitored the influence of these modifications on the intra- and extracellular scFv accumulation of a secretory version of scFv 21C5. Possible quality control mechanisms involved in retention of secretory scFvs in the ER are discussed.

Materials and methods

Cell lines, strains and cloning vectors.

Cloning procedures were according to Sambrook *et al.*, (1989) using the *Escherichia coli* strains DH5α, TG1 and 190 and the scFv cloning vectors pNEM5 and pNEM5K (Schouten *et al.*, 1996).

To obtain vectors for transient expression assays in tobacco the expression cassette from pCPO31 (van Engelen *et al.*, 1994), containing the doubled CaMV 35S promoter, Almv untranslated leader, and Tnos terminator, was cloned into pUCAP (Engelen *et al.*, 1995). The resulting vector pUCAP35S was provided with a kappa signal peptide (van Engelen *et al.*, 1994). Standard scFv cassettes with and without carboxy terminal KDEL extensions, obtained from inserts in the vector pNEM5 and pNEM5K, were inserted in pUCAP35S. In the thus obtained vectors pRAP35-S (Secretory) and pRAP35-SK (Secretory KDEL) scFv encoding regions can be cloned as *SalI*/*SmaI* or *SalI*/*NotI* fragments between the kappa signal peptide and the c-myc tag or c-myc tag plus C-terminal KDEL extension.

For transformation into tobacco (*N. tabacum* cv. Samsun NN) the system based on the binary vector pBINPLUS (van Engelen *et al.*, 1995) was used.

ScFv modifications.

To modify the 202' derived peptide linker of the scFv21C5 anti-cutinase the V_L domain was reamplified using the primers L5d (5'-GGTGTCGACGGTGATGTTKTGATGACCCAAA-3') and Vkglyc (5'-AGCCGGATCCGTTGGATTTACCCTCGAGTTTTATTTCCARCTTKGTSCC-3').

Restriction sites in the primers, used in the cloning procedure, are underlined. PCR conditions were used as described by Schouten *et al.*, (1996). The amplified fragment was inserted into the *SalI*/*Bam*HI restricted vector pNEM5. The resulting $scFv^{LNC}$ (Fig. 1A, superscript refers to the modified linker residues at positions 1, 6 and 16 in single amino acid code) contains a linker flanked by *Xho*I sites. With respect to the original scFv ($scFv^{RSC}$) the Arg at position 1 of the linker is replaced by Leu. The Ser at position 6 by Asn, thereby creating a consensus site for N-linked glycosylation (Kornfeld and Kornfeld, 1985). A control scFv construct in which the Asn is replaced by Ser, but the Leu at position 1 retained, was obtained by inserting an *XhoI*/*Bam*HI adapter fragment (5'-TCGAGGGTAAATCCTCCG-3' and 5'-GATCCGGAGGATTTACCC-3'). This scFv is referred to as $scFv^{LSC}$ (Fig. 1A).

In order to replace the Cys in the linker of the $scFv^{RSC}$ by Ser, the *Xho*I site *Pvu*II fragment was replaced by a suitable adapter (5'-TCGAGTCTGAGGTCCAG-3' and 5'-CTGGACCTC-

AGAC-3') resulting in the $scFv^{RSS}$. The $scFv^{LNS}$ and $scFv^{LSS}$ were constructed by exchanging *SalI/Bam*HI fragment of $scFv^{RSS}$ with the same fragments of $scFv^{LNC}$ and $scFv^{LSC}$ (Fig. 1A).
To delete the five amino acids of the C_H1 domain the $scFv^{RSS}$ was reamplified with the primers L5d and 3FvH (5'-CTCCCCGGGTGARGAGACDGTGAS-3') and cloned as *SalI/SmaI* fragment in pNEM5 resulting in the $scFv^{\Delta CH1}$ (Fig. 1A).
To construct a scFv 21C5 with $(Gly_4Ser)_3$ peptide linker in the V_L-V_H and V_H-V_L architecture the original scFv construct ($scFv^{RSC}$) was reamplified with a suitable set of primers. For the $scFv^{L(gs)H}$ the V_L domain was amplified by the primers L5d and 3KGS (5'-ACCGGATCC-TCCACCTCCAGAACCACCACCCCCTTTTATTTCCARCTTKGTSCC-3'). For the V_H domain the combination of primers 5HGS (5'-GGTGGAGGATCCGGTGGAGGAGGTTCTGAGGTYCAGCTG-CARSA-3') and VH33b (5'-GCACGTTAACCCCGGGTGTTGTTTTGGCTGCAGAGACAG-3') was used. Fragments were cloned jointly into pNEM5 and the plant cloning vectors pRAP35-S and pRAP35-SK (Fig. 1B).
The $scFv^{H(gs)L}$ was cloned similarly from amplification products of the V_H domain reamplified with primers H5c (5'-CGGTGTCGACTCCGAGGTCCAGCTGCAACARTC-3') and 3HGS (5'-ACCGGATCCACCACCACCAGATCCCCCTCCGCCTGARGAGACDGTGAS-3') and the V_L domain reamplified with primers 5Lgs (5'-GGTGGTGGATCCGGTGGAGGTGGTTCTGATGTTGTGATG-ACCCAAACT-3') and 3K (5'-TCCCCCGGGTTTTATTTCCARCTTKGTSCC-3') (Fig. 1B).
To obtain an scFv antibody fragment with short hinge the $scFv^{L(gs)H}$ gene construct was reamplified using L5d as 5'-end primer and 3HSH (5'-TCCCCCGGGGCACTTTGGGCACT-TGCAGACCTCGTTAGTTCCTGA(G/A)GAGAC(T/A/G)GTGA(C/G)-3') as 3'-end primer. This $scFv^{L(gs)H}$-SH gene construct (Fig. 1C) was cloned as *SalI/SmaI* fragment in *SalI/SmaI* digested pRAP35-S and pRAP35-SK.
To obtain an scFv antibody fragment with long hinge the $scFv^{L(gs)H}$ gene construct was reamplified using L5d as 5'-end primer and 3HLH (5'-TCCCCCGGGGAATCTTTGGCTCT-GA(A/G)GAGAC(T/A/G)GTGA(G/C)-3') as 3'-primer. This fragment was *SalI/SmaI* digested and ligated to the blunt/*NotI* long hinge fragment (LH1-4), resulting in the $scFv^{L(gs)H}$-LH gene construct. The LH1-4 fragment was constructed out of four oligonucleotides, LH1 (5'-AAC-CACAACCTAAGCCACAACCACAACCTCAACCACAACCTAAG-3') and LH2 (5'-pCCACAA-CCTAAGCCAGAGCCTGAGTGCACTTGCCCTAAGTGCCCAGC-3') for the plus strand, and LH3 (5'-GGCCGCTGGGCACTTAGGGCAAGTGCACTCAGGCTCTGGCT-3') and LH4 (5'-pTAGGTTGT-GGCTTAGGTTGTGGTTGAGGTTGTGGTTGTGGCTTAGGTTGTGGTT-3') for the minus strand. The $scFv^{L(gs)H}$-LH gene construct (Fig. 1C) was cloned as in *SalI/NotI* digested pRAP35-S and pRAP35-SK.
To construct the $scFv^{L(gs)H}$, $scFv^{L(gs)H}$-SH and $scFv^{L(gs)H}$-LH genes with C_H4 domain $poly(A)^+$ RNA from an IgM producing hybridoma cells was isolated using the QuickPrep Micro mRNA purification kit (Pharmacia) and cDNA was synthesized using the Pharmacia First Strand cDNA Kit. The C_H4 domain was amplified using the 5CH4 (5'-CCCAGCGGCCGCACCCAG-GAATGAGGTGCACAAACATCCA-3') as 5'-end primer and H3M (5'-GTGCGTTAACCCCGG-GTTAATAGCAGGTGCCGCCTGTGTC-3') as 3'-end primer. This C_H4 domain was subcloned as *NotI/HpaI* fragment in *NotI/Eco*RV digested pNEM5, resulting in pNEM5-C_H4. Next the C_H4 domain was isolated as *NotI/XbaI* from pNEM5-C_H4 and cloned into *NotI/XbaI* digested pRAP35, resulting in pRAP-C_H4 (Fig. 1C). The $scFv^{L(gs)H}$, $scFv^{L(gs)H}$-SH and $scFv^{L(gs)H}$-LH gene constructs cloned in pRAP35 were isolated as *SalI/NotI* fragments and each cloned in *SalI/NotI* digested pRAP-C_H4 (Fig. 1C).
All constructs were checked by sequencing (Sanger *et al.*, 1977).

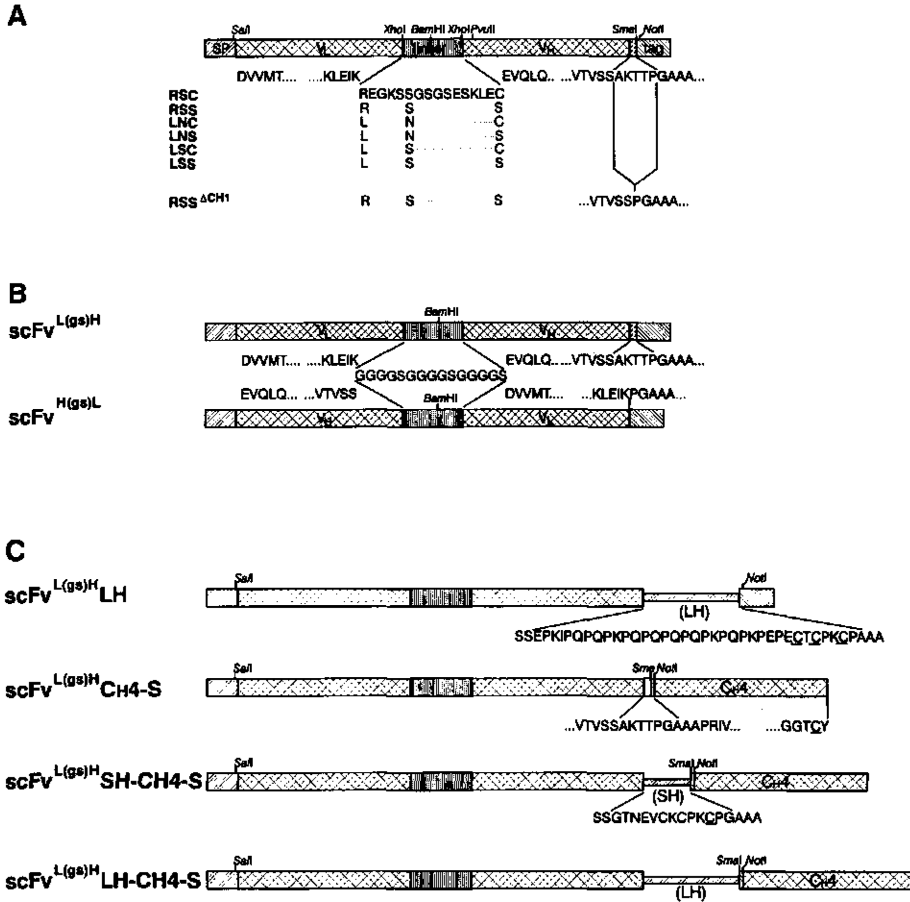

Fig. 1. Schematic representation of the various 21C5 scFv constructs. A, modifications in the 202'-derived linker peptide; B, scFvs with $(Gly_4Ser)_3$ linker peptide; C, scFvs with extra domains for the formation of $F(ab')_2$-like fragments. The amino acid sequences (single letter code) of the linker peptide, the long and short hinges and both the N- and C-terminus of the variable domains are indicated. The different 202' linker variants are named according to the amino acids present at position 1, 6 and 16 in peptide linker. In RSSΔCH1 five amino acids of the C_H1 region are removed. The constructs with $(Gly_4Ser)_3$ linker (gs) are named according to the orientation of the V_L (L) and V_H (H) domain. All fragments in panels A and B and the $scFv^{L(gs)H}$LH fragments were constructed in the absence (-S) and presence (-SK) of the ER retention signal of KDEL. Abbreviations: SP, signal peptide; V_L, variable domain of the light chain; V_H, variable domain of the heavy chain; C_H1, first constant domain of the heavy chain; LH, camel IgG_2 long hinge; SH, camel IgG_3 short hinge; C_H4, mouse IgM C_H4 domain; tag, c-myc tag.

Tobacco transformation and protein analysis.
The scFv$^{L(gs)H}$-S and -SK and scFv$^{L(gs)H}$-LH-S and -SK expression cassettes, cloned in pRAP35-S and pRAP35-SK, and the scFv$^{L(gs)H}$-C_H4-S and scFv$^{L(gs)H}$-LH-C_H4-S, cloned in pRAP35-S, were isolated as *PacI-AscI* fragments and transferred to the binary vector pBINPLUS as described by van Engelen *et al.* (1995). The resulting plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983). Tobacco transformation was conducted according to Horsch *et al.* (1985). Kanamycin resistant transformants were regenerated and total soluble proteins were extracted by grinding leafs essentially as described by Schouten *et al.* (1996). The proteins were analyzed by loading 30 µg total soluble protein homogenate on an SDS-polyacrylamide gel followed by electro blotting and immundetection as described.

Preparation of protoplasts and transient gene expression.
Protoplasts were obtained from leafs of untransformed and transformed tobacco plants (*N. tabacum* cv. Samsun) by the method described by Denecke *et al.* (1989).
For transient expression assays DNA was introduced into untransformed tobacco protoplasts according to the polyethylene glycol procedure (Denecke *et al.*, 1989). To study the scFv glycosylation *in vivo* the transfected protoplasts were incubated in TEX medium supplemented with 10 µg/ml tunicamycin (Iturriaga *et al.*, 1989).
The protoplasts were separated from the incubation medium and lysed by adding an equal volume of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 1 mM Pefabloc SC (Boehringer), and subsequent vortexing. The chloroplasts were pelleted by centrifugation at 13000×g for 2 min and the supernatant was used for further analysis.
For the secretion experiments the protoplasts from individual stable transformants expressing scFv$^{L(gs)H}$-LH-SK, scFv$^{L(gs)H}$-C_H4-S, scFv$^{L(gs)H}$-LH-C_H4-S proteins were isolated and resuspended in TEX medium to a concentration of 2×10^6 protoplasts/ml. 500µl protoplast suspension expressing scFv$^{L(gs)H}$-LH-SK was mixed with 500µl protoplasts expressing scFv$^{L(gs)H}$-C_H4-S or scFv$^{L(gs)H}$-LH-C_H4-S and incubated at 25°C for 72 hours. Where indicated the TEX medium contained 10 µg/ml tunicamycin, 4 mM β-mercaptoethanol (2-ME), or 10 mM dithiothreitol (DTT).
The protoplasts were separated from the incubation medium and lysed by adding an equal volume of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM Pefabloc, 80 mM N-ethylmaleimide (NEM) and subsequent vortexing. The suspension was kept at 0°C for 5' and at room temperature for 10'. The chloroplasts were pelleted by centrifugation and the supernatant was isolated. For western analysis to the medium samples NEM was added to a final concentration of 40 mM. The solution was kept at 0°C for 5 min and at room temperature for 10 min.

Protein analysis.
For western analysis one third volume of 4×SDS-PAGE sample buffer, containing 244 mM Tris-HCl, pH 6.8, 8% (w/v) SDS, 50% (w/v) glycerol and 1 mM Pefabloc SC (Boehringer), was added to an aliquot of the supernatant, equalling 3×10^4 to 5×10^4 protoplasts. The secreted proteins were precipitated by adding 3 volumes ethanol to the TEX medium, equalling 3×10^4 to 5×10^4 protoplasts, 30 min incubation at -80°C, and centrifugation at 13000×g for 15 min. The protein pellets were dissolved in 1×SDS-PAGE sample buffer. The samples were incubated at 100°C for 5 min and bromophenolblue was added to a final concentration of 0.008% (w/v).

For deglycosylation samples from protoplasts or medium, equalling 3×10^4 to 5×10^4 were denatured by adding SDS and β-mercaptoethanol to final concentrations of 0.5% (w/v) and 0.2% (v/v), respectively, and incubation at 100°C for 5 minutes. The proteins were deglycosylated using PNGase F (500 units/μl, New England Biolabs) or endo H_f (1000 units/μl, New England Biolabs) according to the manufacturer's protocol. As a control a mock treated sample, in which water replaced the enzyme, was included in the assay. The samples were incubated at 37°C for 2 hrs and bromophenolblue was added to a final concentration of 0.008% (w/v).

Aliquots equalling $3x10^4$ to $5x10^4$ protoplasts were loaded on a 13% SDS-polyacrylamide gel (Leammli, 1970; Bio-Rad mini protean system). After electrophoresis the proteins were transferred to a PVDF membrane (Millipore) by electro blotting. For immunodetection the membranes were incubated with 9E10 monoclonal antibody (1 μg/ml) (Munro and Pelham, 1986), followed by rat-anti-mouse alkaline phosphatase conjugate (0.2 μg/ml, Jackson Immuno Research). Alternatively, the scFv proteins were detected using a rabbit polyclonal anti-21C5 serum, precleared from antibodies reacting to the constant domains, followed by a goat-anti-rabbit alkaline phosphate conjugate (0.2 μg/ml, Jackson Immuno Research).C_H4 domains were detected using a anti IgM alkaline phosphate conjugate (0.2 μg/ml, Southern Biotechnology Associates Inc.). The blots were stained in 0.1 M ethanolamine-HCl pH 9.6, supplemented with 4 mM MgCl2, 5-bromo-4-chloro-3-indolyl phosphate (0.06 mg/ml) and nitro blue tetrazolium (0.1 mg/ml). The relative molecular weights of the proteins were estimated with pre-stained low or broad range molecular weight markers (Bio-Rad).

Quantification of scFv.

To analyze the functional binding of the different scFv versions, an ELISA was carried out as described previously (Schouten *et al.*,1996). To enable the quantification of the ELISA readings a dilution series of known amounts of purified bacterially expressed $scFv^{RSC}$ was loaded in the same experiment. After correction for background, the ELISA readings were converted to nanograms by interpolation.

The expression levels of the different constructs were compared by statistical analysis using the Mann-Whitney test or the Wilcoxon matched-pairs test.

Results.

Modifications in the 202'-derived linker peptide.

If poor expression of scFv molecules is a consequence of the amino acid sequences introduced in the cloning procedure, modifications of these features might allow us to find factors that could improve expression. The modifications were introduced into a model scFv construct (Fig. 1, A and B). Replacing the Cys by a Ser is expected to prevent the formation of dimers. Introduction of the LNS motif is expected to result in N-linked glycosylation. Substitution of the Arg by Leu may prevent proteolysis in the endoplasmic reticulum (ER). Both secretory versions as well as KDEL versions were constructed. The latter allow retention in the ER and were used as controls since addition of the C-terminal KDEL sequence results in high intracellular accumulation (Schouten *et al.*, 1996 and 1997). All constructs were tested in transient expression assays. The effect of the different modifications was assayed by SDS-PAGE followed by immunoblotting using the anti-c-myc

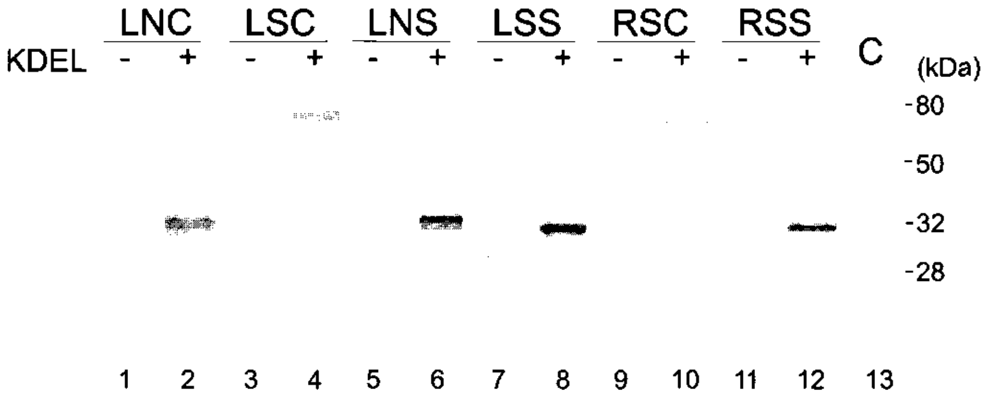

Fig. 2. Western blot analysis of non-reduced protein extracts of tobacco protoplasts transfected with the different 202'-derived linker constructs with (+) and without (-) C-terminal KDEL extension. For detection the anti-c-myc antibody was used. C, mock transfected protoplasts.

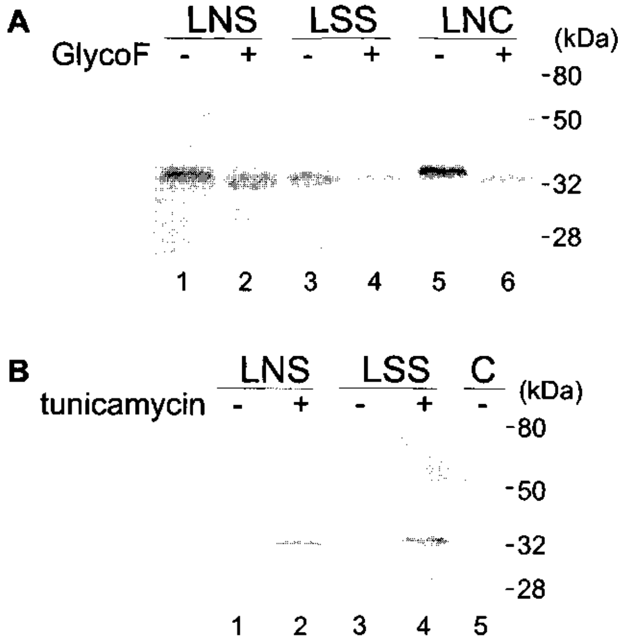

Fig. 3. Analysis of glycosylation state of scFv molecules in protoplasts, transiently expressing scFv-SK constructs with modified 202'-derived linkers. Panel A: Western blot analysis after incubation in the presence (+) or absence (-) of tunicamycine. C, mock transfected protoplasts. Panel B: Western blot analysis after treatment (+) or mock treatment (-) of the protein samples with N-glycosidase F (GlycoF). Proteins were detected using the anti-c-myc antibody.

tag antibody 9E10 as probe to visualize expressed proteins. A typical western blot of the intracellular accumulation of the different scFv molecules is shown in Figure 2. The different SK-versions clearly accumulate intracellularly. In contrast, the S-versions are hardly detectable. For both the scFv-SK and the scFv-S versions the amounts present in the medium fall below the detection limit for western blot analysis. This lack of secretion is not a property of the tobacco protoplasts since transfection with full size antibody constructs resulted in secretion (not shown).

Modified single chain antibody fragments targeted to the secretory pathway.
To address the question where in the secretory pathway breakdown takes place we studied the expression of the scFv-SK constructs having the Asn-X-Ser consensus sequence for N-linked glycosylation in the linker, as depicted in Fig. 1A. The presence of this sequence caused both $scFv^{LNS}$-SK and $scFv^{LNC}$-SK molecules to migrate as 33 and 32 kDa bands (Fig. 2A, lanes 2 and 6). Upon treatment with N-glycosidase F these molecules migrated solely as a 32 kDa product, whereas the mobility of $scFv^{LSS}$-SK was not affected (Fig. 3A). Similar observations were made after endoglycosidase H treatment (results not shown). Results, congruent with these findings were obtained *in vivo* using the glycosylation inhibitor tunicamycin (Fig. 3B). The presence of this compound only induced a shift in migration in the $scFv^{LNS}$-SK and $scFv^{LNC}$-SK (not shown), thus confirming that the asparagine in the linker was indeed glycosylated. Since N-linked glycosylation takes place exclusively in the ER, the experiments also prove that the scFv-SK molecules are translocated to this compartment. In addition, the Endo H sensitivity indicates that these fragments do not reach the medial Golgi or later parts of the secretory pathway.

Intermolecular linker peptide disulfide bonds.
Previously, we concluded that formation of the 65kDa protein is dependent on the presence of the cysteine in the linker and that the product represents a true scFv dimer (see Chapter 5). The state of this cysteine in the linker can therefore be considered as a free cysteine until dimerization occurs. Again, analysis of protoplasts expressing $scFv^{LSC}$-SK and $scFv^{RSC}$-SK showed that the molecule is predominantly migrating at the dimeric position (Fig. 2 lanes 4 and 10) in the absence of the reducing agent DTT. Remarkably, the $scFv^{LNC}$-SK molecules showed no dimerization (Fig. 2, lane 2). Apparently, glycosylation prevents dimerization. Since the four cysteines, supposed to be participating in the intra domain disulfide bonds, are still present in all different linker constructs, this indicates that these cysteines are not involved in the intermolecular disulfide bond.

The scFv constructs with 202' linker variations are functional.
In order to determine the amounts of scFv expressed and to establish functionality an ELISA was carried out using anti-c-myc and anti-Fv antibodies for detection. Assuming that differences in the peptide linker do not substantially influence scFv affinity, comparisons can be made between the different constructs. Statistical analysis showed some significant differences (Table 1). The amount of scFv present in the medium does not differ significantly between the scFv-S and scFv-SK constructs ($p>0.05$; Mann-Whitney) independent of the variations in the linker. The use of an anti-Fv antiserum did not change this figure. Apparently, these results are not influenced by the possible loss of the c-myc tag. This exposed tag is potentially sensitive to digestion by several proteases. Thus, the low apparent secretion levels could be caused by degradation of the scFv in the culture medium. However this is rather unlikely since purified $scFv^{RSC}$ incubated in spent medium is stable during the

Table 1. Expression levels based on functional binding of the scFv molecules carrying different 202' derived linker modifications. (n) is number of experiments

A. Intra (c) and extracellular (m) scFv accumulation of the 202'-derived linker variants represented as ng/3×10^4 protoplasts or equivalent part of the medium.			
		-SER-	-CYS-
RS*	c	7.9 ± 9.4 (6)	5.8 ± 5.5 (7)
	m	0.7 ± 1.1 (6)	1.0 ± 1.0 (7)
LS*	c	1.5 ± 1.2 (4)	2.5 ± 1.8 (3)
	m	0.47 ± 0.67 (4)	0.1 ± 0.1 (3)
LN*	c	9.8 ±11.9 (5)	3.5 ± 1.5 (3)
	m	1.5 ± 1.8 (5)	0.8 ± 0.3 (3)
RS*-KDEL	c	81.6 ± 102.7 (7)	61.9 ± 60.5 (9)
	m	3.0 ± 5.9 (7)	0.9 ± 1.4 (9)
LS*-KDEL	c	39.4 ± 17.2 (2)	24.4 ± 24.8 (3)
	m	0.6 ± 0.1 (2)	0.3 ± 0.4 (3)
LN*-KDEL	c	101.0 ± 114.0 (6)	44.6 ± 32.4 (4)
	m	2.8 ± 3.0 (6)	0.6 ± 0.3 (4)

B. Ratio of intracellular scFv accumulation in absence and presence of KDEL (S/SK)		
	-SER-	-CYS-
1. RS*	0.129±0.090 (9)	0.117±0.062 (6)
2. LS*	0.025±0.009 (2)	0.083 (1)
3. LN*	0.079±0.027 (4)	0.060±0.011 (2)

incubation periods used in the protoplast assays (Fig. 4).
The intracellular scFv accumulation shows a strong effect of the C-terminal KDEL extension (Table 1.). Independent of the linker employed the intracellular accumulation of the scFv-SK molecules is much higher than of the scFv-S ($p<0.001$; Mann-Whitney). Since the variances are rather high it is difficult to establish the influence of the individual changes in the linker. The only difference with significance is the replacement of Cys by Ser, in the scFv-SK, which increases the intracellular scFv accumulation ($0.025< p <0.05$; Wilcoxon). This is in agreement with the results concerning scFv-SK accumulation when analyzed on a western blot (Fig. 2) and could indicate that also in plants a free thiol dependent quality control mechanism of protein assembly is acting.

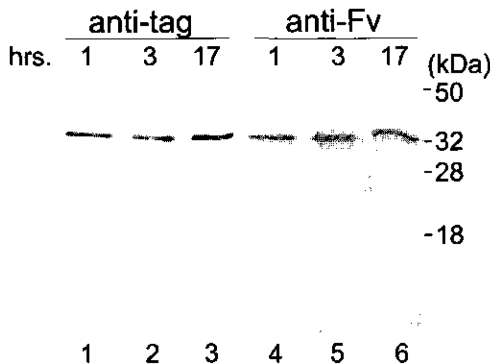

Fig. 4. Western blot analysis to determine stability of purified $scFv^{RSC}$, incubated for 1, 3 and 17 hours in spent protoplast culture medium. $scFv^{RSC}$ was detected using the anti-c-myc tag and anti-Fv antisera.

Expression of scFv 21C5 with $(Gly_4Ser)_3$ linker and deleted C_H1 residues.
The low intra- and extracellular accumulation of the scFv-S constructs prompted us to investigate whether the often used $(Gly_4Ser)_3$ linker as well as the scFv domain order (V_L-V_H versus V_H-V_L) would improve expression. Since the $scFv^{H(gs)L}$ does not carry the five amino acid residues of the C_H1 region the influence of this change was also studied in a scFv with 202' linker in the V_L-V_H architecture ($scFv^{RSS\Delta CH1}$). For comparison the 202' linker construct ($scFv^{RSS}$) was included. Medium samples from protoplasts transfected with the different constructs did not show any signal on western blots indicating that secretion of the scFv was not improved. Immunoblotting of the intracellular proteins using the anti-c-myc antibody revealed that, again, only the constructs with KDEL had detectable expression levels (Fig. 5, panel A). In contrast, after decorating the blot with the anti-Fv antiserum, the $scFv^{LgsH}$-S was detectable at an expression level similar to that observed with scFv-SK (Fig. 5, panel B, lane 3). This material migrated predominantly as a 30 kD product, indicating that the c-myc tag was cleaved off. It is noteworthy that this only applied for the V_L-V_H configuration. The increased intracellular accumulation indicates that the $(Gly_4Ser)_3$ linker improves the scFv stability. The absence of the five amino acids of the C_H1 did not influence scFv expression since the amount observed were not different for the $scFv^{RSS}$ and $scFv^{RSS\Delta CH1}$ (Fig. 5, lanes 5 to 8). In all, this implies that the difference in expression between the V_L-V_H and V_H-V_L

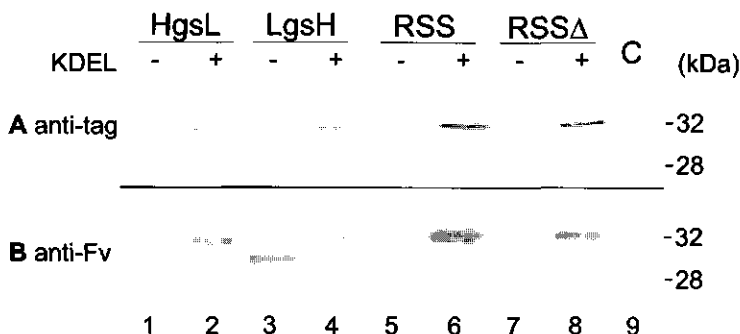

Fig. 5. Western blot showing intracellular accumulation in transfected tobacco protoplasts of the secretory (-KDEL) and ER retained (+KDEL), $scFv^{H(gs)L}$ (HgsL), $scFv^{L(gs)H}$ (LgsH), $scFv^{RSS}$ (RSS) and $scFv^{RSS\Delta CH1}$ (RSSΔ) under non-reducing conditions, detected by the anti-c-myc tag (panel A) and anti-Fv (panel B) antisera.

configuration results from the domain orientation and not from the presence of the constant domain residues.

The functional analysis of these constructs based on ELISA was well in agreement with the results observed on western blots (Table 2). Secretion did not differ for the scFv-S and scFv-SK constructs ($p > 0.05$; Mann-Whitney), independent of the antibody (anti-Fv or anti-c-myc) used for detection. However, as compared to the 202' linker constructs, the $(Gly_4Ser)_3$ linker had a significant effect on the intracellular accumulation of functional scFv in the scFv-S format ($0.01 < p < 0.025$; Mann-Whitney). The amount of functional scFv in the V_L-V_H configuration does reach a level similar to that observed for the scFv-SK constructs.

The reverse domain orientation resulted only in minute amounts of functional $scFv^{H(gs)L}$ in lower ELISA readings and deduced scFv amounts (Table 2.).

Expressed $scFv^{H(gs)L}$ constructs *with camel hinges and/or IgM C_H4 domain can dimerize.*

The previous results showed that the choice of linker, the architecture and the residues present at the C-terminus do not improve scFv secretion. Apparently, the structural properties of the variable domains dominate in determining whether transport through the secretory pathway is attenuated. Quality control mechanisms for protein export present in the secretory pathway may be responsible for this. This might be overcome by the addition antibody constant domains and camel antibody hinges, which would also mediate dimerization.

The IgM C_H4 domain or the camel IgG_2 long hinge (LH) were added C-terminally to $scFv^{L(gs)H}$ antibodies (Fig. 1C). In addition, we constructed $scFv^{L(gs)H}$ genes carrying a LH or camel IgG_3 short hinge (SH) coding sequence combined with the C_H4 coding sequence. The influence on both overall protein stability in the secretory pathway and secretion were studied. We chose the $scFv^{L(gs)H}$ since the transient assay indicated that this antibody fragment remained relatively stable intracellularly in the absence of the ER retention signal.

The $scFv^{L(gs)H}$ with long hinge was constructed as both a secretory fragment ($scFv^{L(gs)H}$-LH-S) and ER retained fragment ($scFv^{L(gs)H}$-LH-SK).

Table 2. Intra (c) and extracellular (m) scFv accmulation of the constructs with gly-ser linker and ΔCH1 (ng/3×10^4 protoplasts)

		a-Tag	a-Fv
LgsH	c	6.67 ± 9.73 (4)	32.7 ± 58.1 (4)
	m	1.00 ± 1.08 (3)	1.52 ± 2.32 (3)
LgsH-KDEL	c	66.1 ± 72.2 (6)	34.1 ± 52.2 (6)
	m	1.76 ± 2.15 (4)	0.94 ± 1.43 (4)
HgsL	c	0.42 ± 0.49 (4)	1.93 ± 2.66 (4)
	m	0.13 ± 0.16 (4)	0.71 ± 1.26 (4)
HgsL-KDEL	c	28.9 ± 25.0 (5)	8.35 ± 7.75 (5)
	m	0.37 ± 0.51 (4)	0.29 ± 0.33 (4)
RSS	c	2.39 ± 3.41 (5)	2.37 ± 3.63 (5)
	m	0.68 ± 1.23 (5)	0.72 ± 1.30 (5)
RSS-KDEL	c	77.3 ± 139.8 (6)	49.9 ± 102.0 (6)
	m	4.32 ± 8.15 (6)	2.49 ± 4.39 (6)
ΔCH1	c	3.73 ± 5.95 (3)	4.64 ± 7.76 (3)
	m	0.99 ± 1.68 (3)	0.35 ± 0.48 (3)
ΔCH1-KDEL	c	114.8 ± 190.7 (3)	45.4 ± 76.7 (3)
	m	2.55 ± 3.42 (3)	0.61 ± 0.74 (3)

In western blot analysis under non-reducing conditions of protoplasts transfected with both scFv$^{L(gs)H}$-LH-S and scFv$^{L(gs)H}$-LH-SK constructs two protein bands were detected intracellularly with estimated sizes of 38 and 75 kDa when using anti-Fv antiserum (Fig. 6, lanes 4 and 5). The scFv$^{L(gs)H}$-LH-SK protoplasts also showed a weaker band of approximately 57 kDa, which probably represents a degradation product of the 75 kDa band.No protein bands were observed when using anti-IgM. Using anti-Fv or anti-IgM antiserum for the scFv$^{L(gs)H}$-C_H4-S construct protein bands, estimated at 42, 45 and 90 kDa, were detected (Fig. 6, lane 6). For the scFv$^{L(gs)H}$-LH-C_H4-S protein bands estimated at 46, 50 and 100 kDa and two distinct but faint bands in the range of 150 to 200 kDa (Fig. 6, lane 7) were found. Expressing scFv$^{L(gs)H}$-SH-C_H4-S resulted in the detection of 47 and 95 kDa

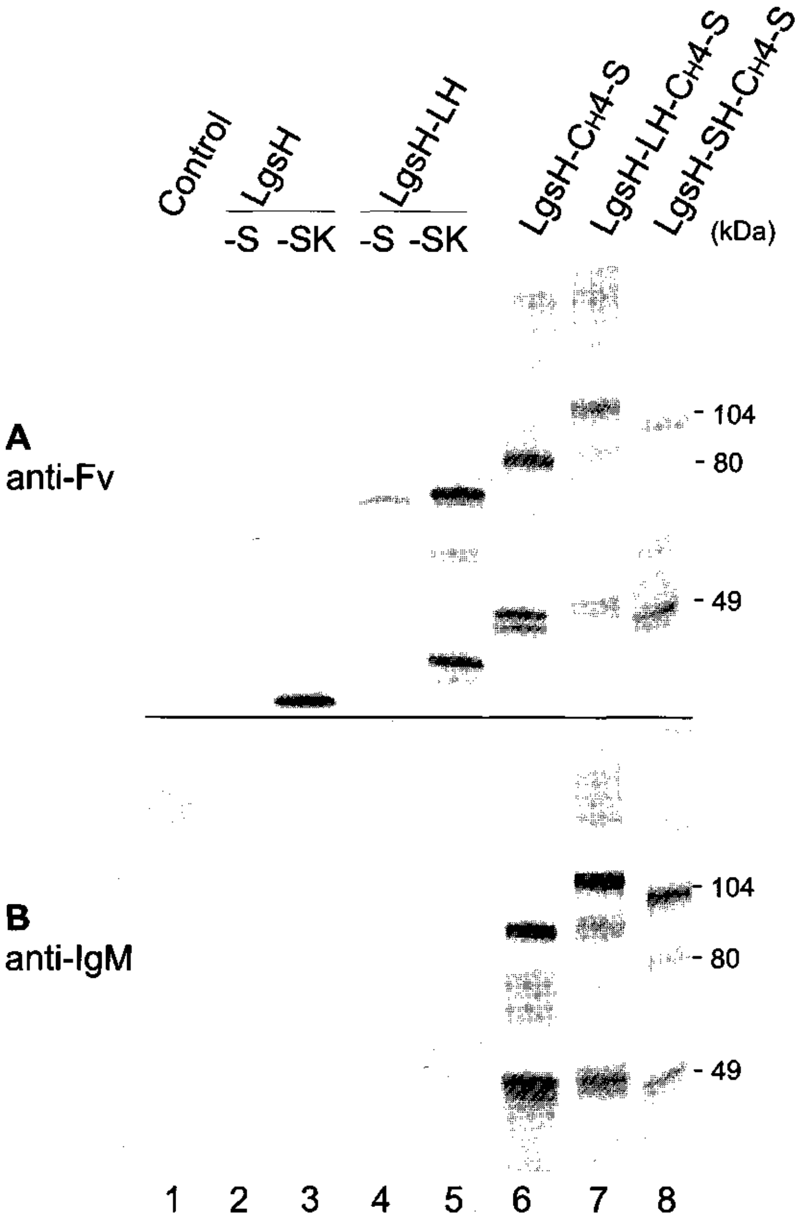

Fig 6. Western blot showing intracellular accumulation in transfected tobacco protoplasts of the secretory (-S) and ER retained (-SK) scFv$^{L(gs)H}$ (lanes 2 and 3), scFv$^{L(gs)H}$-LH (lanes 4 and 5) and the secretory scFv$^{L(gs)H}$-C_H4-S (lane 6), scFv$^{L(gs)H}$-LH-C_H4-S (lane 7) and scFv$^{L(gs)H}$-SH-C_H4-S (lane 8) under non-reducing conditions, detected by the anti-c-myc tag (panel A) and anti-IgM (panel B) antisera.

bands. These results show that the secretory scFv constructs can accumulate intracellularly as a non-degraded protein.
Under reducing conditions only the 38 kDa (scFv$^{L(gs)H}$-LH-SK), the 46 kDa (scFv$^{L(gs)H}$-C$_H$4-S), the 50 kDa (scFv$^{L(gs)H}$-LH-C$_H$4-S) and 47 kDa(scFv$^{L(gs)H}$-SH-C$_H$4-S) bands were detected (Fig. 7, lanes 5, 6 and 7). As shown previously, no scFv$^{L(gs)H}$-S protein and the 32 kDa scFv$^{L(gs)H}$-SK protein were detected when using the anti c-myc antibody (Fig. 7, lanes 1 and 2). Apparently, although some protein degradation occurs the ER retained scFv$^{L(gs)H}$-LH-SK and secretory scFv$^{L(gs)H}$-LH-S, scFv$^{L(gs)H}$-C$_H$4, scFv$^{L(gs)H}$-SH-C$_H$4 and scFv$^{L(gs)H}$-LH-C$_H$4 are synthesized and a large fraction (up to approximately 70%) forms dimers.
Since both the LH and the C$_H$4 contain free cysteines for disulfide bridge formation it is, in theory, possible that the scFv$^{L(gs)H}$-LH-C$_H$4-S dimers themselves can form multimers, possibly even up to IgM-like pentamers. The results obtained with scFv$^{L(gs)H}$-LH-C$_H$4-S suggests that this is happening. Since the blotting efficiency of high molecular weight proteins is very poor, the results may give a significantly distorted impression of the percentage of these possible high molecular multimers. However, if these multimers are being formed the percentage is rather low since intensity of scFv$^{L(gs)H}$-LH-C$_H$4-S protein band under reducing conditions is not dramatically increased when compared to the intensity of the scFv$^{L(gs)H}$-LH protein band (Fig. 7, lanes 5 and 6).

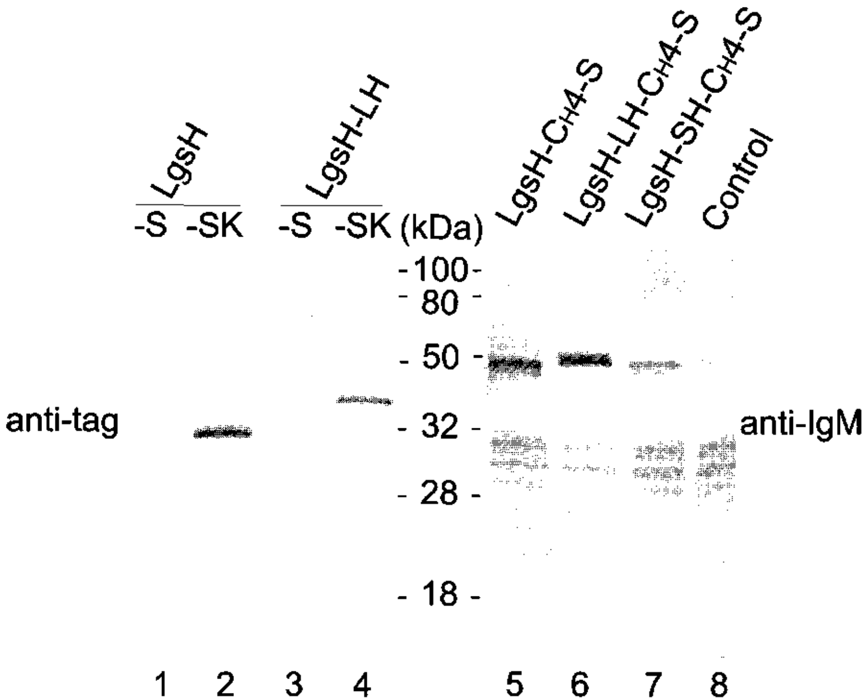

Fig. 7. Western blot showing intracellular accumulation in transfected tobacco protoplasts of the secretory (-S) and ER retained (-SK) scFv$^{L(gs)H}$ (LgsH), scFv$^{L(gs)H}$-LH (LgsH-LH) and the secretory scFv$^{L(gs)H}$-C$_H$4-S (LgsH-C$_H$4-S), scFv$^{L(gs)H}$-LH-C$_H$4-S (LgsH-LH-C$_H$4-S), scFv$^{L(gs)H}$-SH-C$_H$4-S (LgsH-SH-C$_H$4-S) under reducing conditions, detected by the anti-c-myc tag (panel A) and anti-IgM (panel B) antisera.

Expression of scFvs in stable transformed tobacco plants.

The transient expression experiments reported in the previous section suggested that the modified $scFv^{L(gs)H}$ constructs were promising with respect to protein expression level and stability. Therefore, stable tobacco transformants were generated expressing the $scFv^{L(gs)H}$-S, $scFv^{L(gs)H}$-SK, $scFv^{L(gs)H}$-LH-S, $scFv^{L(gs)H}$-LH-SK, $scFv^{L(gs)H}$-C_H4-S and $scFv^{L(gs)H}$-LH-C_H4-S, and analyzed.

The presence of the scFv proteins in the individual transgenics was determined by western blot analysis of leaf extracts under non-reducing conditions. Using anti-Fv antiserum in 50% of the screened $scFv^{L(gs)H}$-S transgenics, a protein band of 30 kDa was observed, whereas in all of the screened $scFv^{L(gs)H}$-SK transgenics a protein band of 32 kDa was found (Fig. 8, lanes 1 and 2). Like in the transient expression assay, the 30 kDa band could not be detected with anti-c-myc antiserum (results not shown) indicating that the c-myc tag is cleaved off.

In 33% of the $scFv^{L(gs)H}$-LH-S transgenics screened two weak protein bands of 75 and 65 kDa could be observed, which were undetectable with anti-c-myc (not shown), whereas all of the $scFv^{L(gs)H}$-LH-SK transgenics showed a strong protein band of 75 kDa and a weaker protein band of 38 kDa (Fig. 8, lanes 3 and 4), which both were also detectable with anti-c-myc (not shown).

In all of the $scFv^{L(gs)H}$-C_H4-S and $scFv^{L(gs)H}$-LH-C_H4-S transgenics screened, protein bands of 45 and 90 kDa and of 50 and 100 kDa were detected, respectively (Fig. 8, lanes 5 and 6).

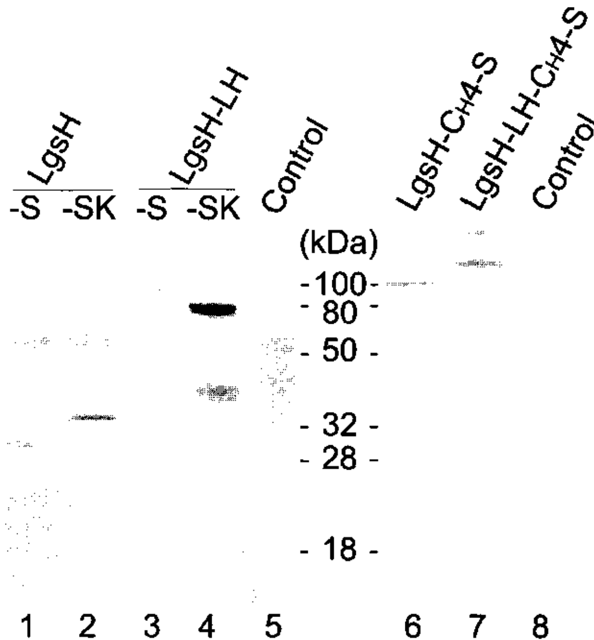

Fig. 8. Western blot analysis of leaf homogenates from stable tobacco transformants expressing the secretory (-S) and ER retained (-SK) $scFv^{L(gs)H}$ (LgsH), $scFv^{L(gs)H}$-LH (LgsH-LH) and the secretory $scFv^{L(gs)H}$-C_H4-S (L(gs)H-C_H4-S), $scFv^{L(gs)H}$-LH-C_H4-S (L(gs)H-LH-C_H4-S) under non-reducing conditions, detected by the anti-Fv antiserum.

The same bands were detected with anti-IgM antiserum (not shown). The highest expression level for both scFv constructs was estimated at 0.2% of total soluble protein.
All these results are congruent with those obtained with the transient expression assays, although the intracellular degradation of $scFv^{L(gs)H}$-LH-S is more distinct in stable transformants.

ScFv$^{L(gs)H}$-LH-C_H4 constructs are secreted.
Next, the efficiency of $scFv^{L(gs)H}$-C_H4-S and $scFv^{L(gs)H}$-LH-C_H4-S secretion by the transformed plants was analyzed. To determine secretion protoplasts were prepared from the $scFv^{L(gs)H}$-C_H4-S, $scFv^{L(gs)H}$-LH-C_H4-S and $scFv^{L(gs)H}$-LH-SK synthesizing transgenics (Fig. 8, lanes 6, 7 and 4 , respectively). Since $scFv^{L(gs)H}$-LH-SK is retained in the ER, it can be used as a control to determine whether all protein detected in the medium was actually secreted by living protoplasts or released by cell disruption and degradation. The $scFv^{L(gs)H}$-LH-SK contains the c-myc tag, whereas both the $scFv^{L(gs)H}$-C_H4-S and $scFv^{L(gs)H}$-LH-C_H4-S lack the c-myc tag but contain the C_H4 domain. By using the proper antibodies for detection, anti-c-myc or anti-IgM, it is possible to discriminate between the different scFv constructs. Protoplasts expressing $scFv^{L(gs)H}$-LH-SK were mixed in equal numbers with those expressing $scFv^{L(gs)H}$-C_H4-S or $scFv^{L(gs)H}$-LH-C_H4-S and incubated for 72 hrs. Anti-IgM detection on western blot of the protoplasts from the $scFv^{L(gs)H}$-C_H4-S/$scFv^{L(gs)H}$-LH-SK mix showed the 45 kDa $scFv^{L(gs)H}$-C_H4-S monomer (Fig. 9, panel A, lane 1). An additional 32 kDa was found, probably representing a degradation product of $scFv^{L(gs)H}$-C_H4-S. In the same mix a clear 75 kDa band and faint 55kDa band was visible when anti-c-myc detection was used (Fig. 9, panel B, lane 1). With both antisera no proteins were observed in the medium (Fig. 9, lane 4, panel A and B). Thus, during the assay the protoplasts remained intact and no detectable levels of $scFv^{L(gs)H}$-C_H4-S were secreted into the medium.
In protoplasts of the $scFv^{L(gs)H}$-LH-C_H4-S/$scFv^{L(gs)H}$-LH-SK mix the $scFv^{L(gs)H}$-LH-C_H4-S 50 kDa monomer and 100 kDa dimer were detected with anti-IgM (Fig. 9, panel A, lane 2) and the 75 kDa $scFv^{L(gs)H}$-LH-SK dimer together with a faint 55kDa protein band were detected with anti-c-myc (Fig. 9, panel B, lane 2). In the medium the 50 and 100 kDa $scFv^{L(gs)H}$-LH-C_H4-S were detected with anti-IgM (Fig. 9, panel A, lane 5) and no $scFv^{L(gs)H}$-LH-SK protein bands were observed with the anti-c-myc antiserum (Fig. 9, panel A, lane 5). This shows that both the monomeric and dimeric $scFv^{L(gs)H}$-LH-C_H4-S fragments are secreted at detectable levels into the medium.

Intracellular scFv$^{L(gs)H}$-LH-C_H4-S fragments are glycosylated and located in the ER.
As demonstrated, the $scFv^{L(gs)H}$-LH-C_H4-S fragments also accumulated intracellularly. These fragments may be retained early in the secretory pathway. This can be analyzed by establishing the maturation of the N-linked glycosylation of the C_H4 domain. $ScFv^{L(gs)H}$-LH-C_H4-S expressing protoplasts were incubated for 72 hrs and total protein homogenates from protoplasts and medium was treated with N-glycosidase F or endoglycosidase H. The relative migration on western blot of the $scFv^{L(gs)H}$-LH-C_H4-S fragments is indicative for glycosylation. In the mock treated protoplast samples 50 kDa $scFv^{L(gs)H}$-LH-C_H4-S protein was detected using anti-IgM antiserum (Fig. 10, lane 1). In both the glycosidase F and endoglycosidase H treated protoplast homogenates the size was reduced to 48 kDa (Fig. 10, lanes 3 and 2, respectively). In the control, which were protoplasts from transformants stably transformed with the empty expression vector pRAP35, a relatively weak band was visible at 50 kDa in the mock treated sample (Fig. 10, lanes 7). This band was not sensitive to N-

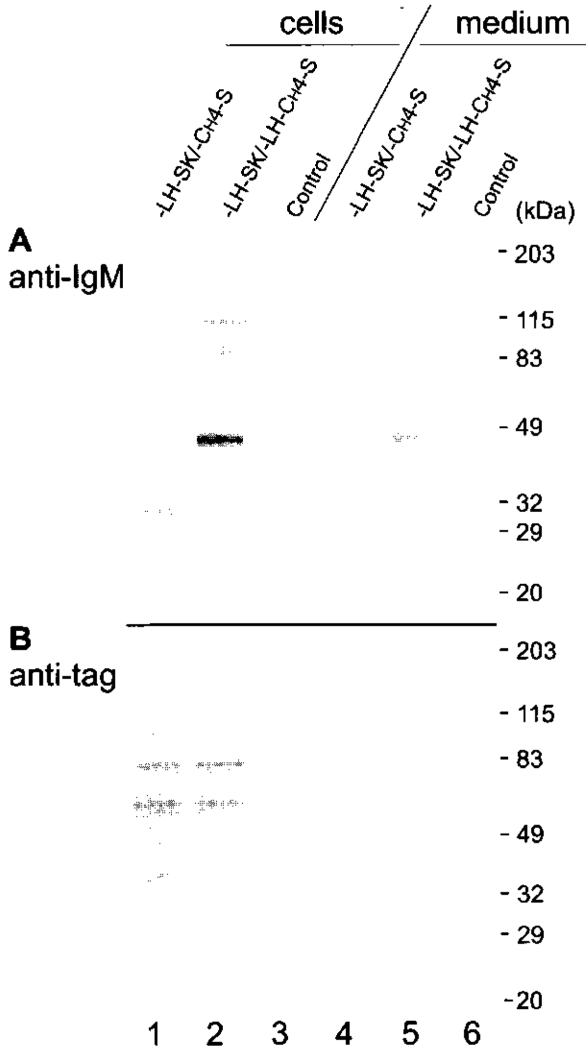

Fig. 9. Location of scFv$^{L(gs)H}$-LH-SK (-LH-SK), scFv$^{L(gs)H}$-C$_H$4-S (-C$_H$4-S) and scFv$^{L(gs)H}$-LH-C$_H$4-S (-LH-C$_H$4-S), analyzed by western blotting. Equal numbers of protoplasts expressing scFv$^{L(gs)H}$-LH-SK and scFv$^{L(gs)H}$-C$_H$4-S (lanes 1 and 4), and scFv$^{L(gs)H}$-LH-SK and scFv$^{L(gs)H}$-LH-C$_H$4-S (lanes 2 and 5) from stable tobacco transformants were mixed and incubated in medium for 72 hrs. As a control protoplasts from transgenic tobacco, stably transformed with the empty expression vector pRAP35, were used (lanes 3 and 6). Protein samples (lanes 1 to 3) and medium (lanes 4 to 6), equaling 5×10^4 protoplasts, were separately analyzed, under non-reducing conditions, for the presence of antibody fragments. For detection the anti-IgM (panel A) or anti-c-myc (panel B) antiserum was used.

glycosidase F or endoglycosidase H treatment (Fig. 10, lanes 8 and 9) and was therefore considered to be aspecific. In the medium sample no significant differences in relative migration of the detected proteins could be found. In the mock treated and enzyme treated samples a protein smear of 50-51 kDa was present. The insensitivity of the scFv$^{L(gs)H}$-LH-C_H4-S present in the medium for N-glycosidase F is unexpected.
These results demonstrate that the scFv$^{L(gs)H}$-LH-C_H4-S fragments are translocated into the secretory pathway and are glycosylated. The Endo H sensitivity indicates that the intracellular scFv$^{L(gs)H}$-LH-C_H4-S fragments are retained in the ER or cis-Golgi prior to secretion.

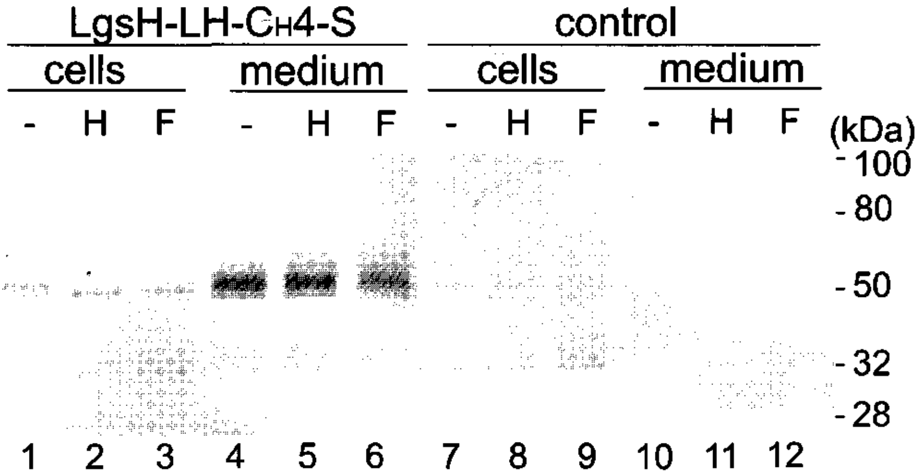

Fig. 10. The glycosylation state of the intracellular (cells) and secreted (medium) scFv$^{L(gs)H}$-LH-C_H4-S expressed by a stable tobacco transformant. Western blot analysis of the protein samples after mock treatment (-) or treatment with endoglycosidase H (H) or N-glycosidase F (F). As a control protoplasts from transgenic tobacco, stably transformed with the empty expression vector pRAP35 were used. Proteins were detected using the anti-IgM antibody.

Secretion of scFv$^{L(gs)H}$-LH-C_H4-S is not primarily regulated by thiol mediated retention.

Secretion of monomeric scFv$^{L(gs)H}$-LH-C_H4-S suggests that these fragments are not retained by thiol mediated quality control mechanism in the ER, which normally prevents the secretion of IgM molecules with free thiols in mammalian cells (Alberini *et al.*, 1990). To determine whether this mechnism still plays some role in the secretion efficiency of monomers and dimers the scFv$^{L(gs)H}$-LH-C_H4-S/scFv$^{L(gs)H}$-LH-SK protoplast mix was incubated in the presence of dithiothreitol (DTT) and β-mercaptoethanol (2-ME), which are known to interfere with this quality control mechanism (Valetti and Sitia, 1994). Analysis of protoplasts and incubation medium on western blot showed, in the presence of 2-ME, no significant reduction in the intracellular and extracellular accumulation of scFv$^{L(gs)H}$-LH-C_H4-S (Fig. 11, panel A, lanes 5 and 6) compared to the control (Fig. 11, panel A, lanes 1 and 2). ScFv$^{L(gs)H}$-LH-SK accumulated only intracellularly and to a slightly higher level, preferentially in the monomeric form (Fig. 11, panel B, lanes 1, 2, 5 and 6). Furthermore, the intensities of the detected protein bands indicate that for both scFv$^{L(gs)H}$-LH-C_H4 and scFv$^{L(gs)H}$-LH-KDEL 2-ME only slightly affects the formation of dimers.

In the presence of DTT accumulation levels of both intracellular and extracellular $scFv^{L(gs)H}$-LH-C_H4-S were significantly reduced (Fig. 11, panel A, lanes 3 and 4). However, the intracellular accumulation level of $scFv^{L(gs)H}$-LH-SK was slightly increased. For both fragments no or little dimer formation was detected. Apparently, DTT induces reduction and partial disassembly of $scFv^{L(gs)H}$-LH-C_H4-S, leading to a reduced stability when passing through the secretory pathway.

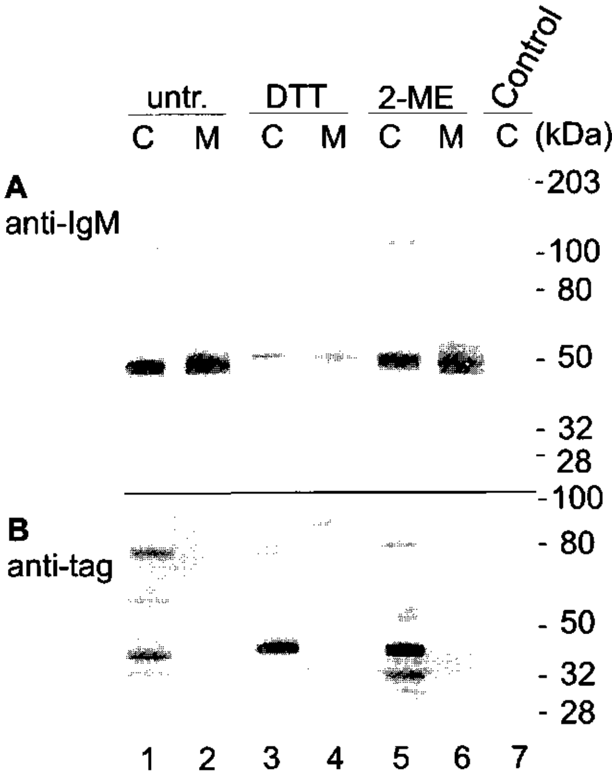

Fig. 11. Influence of reducing agents on the secretion of $scFv^{L(gs)H}$-LH-C_H4-S expressed by protoplasts derived from a stable tobacco transformant, analyzed by western blotting. Equal numbers of protoplasts expressing $scFv^{L(gs)H}$-LH-SK and $scFv^{L(gs)H}$-LH-C_H4-S were mixed and incubated in TEX medium (untr.), or TEX medium supplemented with dithiothreitol (DTT) or β-mercaptoethanol (2-ME), for 72 hrs. As a control protoplasts from transgenic tobacco, stably transformed with the empty expression vector pRAP35, were used (lane 7). The protein samples from cells (C) and medium (M), equaling 5×10^4 protoplasts, were separately analyzed, under non-reducing conditions, for the presence of antibody fragments. For detection the anti-IgM (panel A) or anti-c-myc (panel B) antiserum was used.

Tunicamycin increases $scFv^{L(gs)H}$-LH-C_H4-S secretion.

To establish whether the efficiency in secretion of the $scFv^{L(gs)H}$-LH-C_H4-S fragments is influenced by the degree of glycosylation a $scFv^{L(gs)H}$-LH-C_H4-S/$scFv^{L(gs)H}$-LH-SK protoplast mix was incubated in the presence tunicamycin for 72 hrs. Analysis of protoplasts and medium on western blot showed that in the presence of tunicamycin no $scFv^{L(gs)H}$-LH-SK had

come into the medium (Fig. 12, panel B, lane 4) indicating that, like in the control (Fig. 12, panel B, lane 2), no cell disruption had occurred.

Both incomplete disulfide bridge formation and incomplete glycosylation influences migration into the gel. Therefore, to exclude that possible incomplete disulfide bridge formation caused *the differences in migration the* $scFv^{L(gs)H}$*-LH-*C_H*4-S fragments were analyzed on western* blot under reducing conditions. This showed a reduction in intracellular accumulation and an increase in extracellular accumulation in the presence of tunicamycin when compared to the control (Fig. 12, panel A). The total amount of synthesized $scFv^{L(gs)H}$-LH-C_H4-S was not significantly influenced. Furthermore, the 50 kDa protein band was reduced in intensity whereas the 48 kDa protein band had increased in intensity, both in protoplasts and medium. Apparently, glycosylation of $scFv^{L(gs)H}$-LH-C_H4-S was not completely inhibited by tunicamycin. Although $scFv^{L(gs)H}$-LH-SK contains no sites for N-linked glycosylation intracellular accumulation of this fragment was significantly reduced in the presence of tunicamycin (Fig. 12, panel B). Apparently, tunicamaycin inhibits $scFv^{L(gs)H}$-LH-SK protein synthesis in tobacco protoplasts (Elbein, 1987). In all, the results indicate that the glycosylation process in the ER attenuates $scFv^{L(gs)H}$-LH-C_H4-S transport through the secretory pathway.

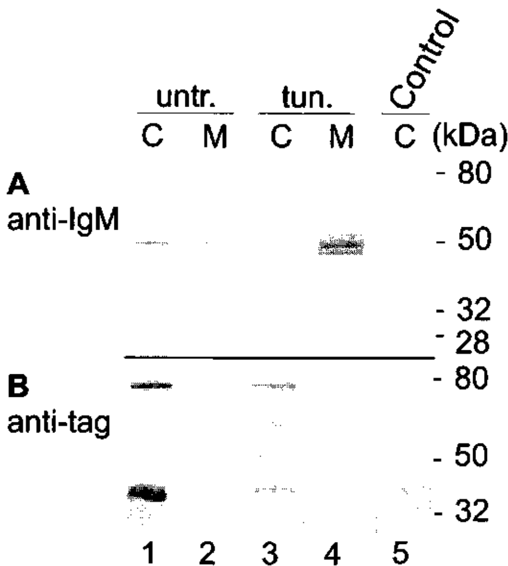

Fig. 12. Influence tunicamycin on the secretion of $scFv^{L(gs)H}$-LH-C_H4-S expressed by protoplasts derived from a stable tobacco transformant, analyzed by western blotting. Equal numbers of protoplasts expressing $scFv^{L(gs)H}$-LH-SK and $scFv^{L(gs)H}$-LH-C_H4-S were mixed and incubated in TEX medium (untr.) or TEX medium supplemented with tunicamycin (tun.) for 72 hrs. As a control protoplasts from transgenic tobacco, stably transformed with the empty expression vector pRAP35, were used (lane 7). Protein samples from cells (C) and medium (M), equaling 5×10^4 protoplasts, were separately analyzed, under non-reducing conditions, for the presence of antibody fragments. For detection the anti-IgM (panel A) or anti-c-myc (panel B) antiserum was used.

Discussion

The expression in plants of secretory single-chain Fv antibody molecules has been less straight forward as could have been expected on the basis of the expression levels obtained with full size antibodies, $F(ab')_2$ and Fab fragments. Apparently the contributions of the constant domains play an important role in obtaining a stable export competent antibody structure. Therefore, with the lack of these contributions expression of secretory scFv molecules could be dominated by the primary amino acid sequence of the variable domains and their compatibility in the final structure (Glockshuber *et al.*, 1990; Rees *et al.*, 1994). However, the final structure of the scFv can also be influenced by the introduced elements needed for scFv construction, like the orientation of the domains, the degree in which they still contain constant region residues and the length and amino acid sequence of the peptide linker used. Since these elements are more amenable to modification than the primary amino acid sequence we have evaluated these factors for the secretory format of the scFv 21C5 (Schouten *et al.*, 1996)

The concept behind the amino acid changes in the 202' derived peptide linker was to decrease its potentially proteolytic sensitivity thereby preventing domain dissociation and degradation (Glockshuber *et al.*, 1990). Removal of the dibasic amino acid sequence Lys-Arg which is recognized by KEX-like proteases in the ER (Julius *et al.*, 1984; Hein *et al.*, 1991) did not influence the accumulation and secretion of the scFv. The same was observed for the expression of individual light and heavy chains of full size antibodies (Hiatt *et al.*, 1993). Apparently, these sites are, even in the scFv format, buried in the folded structure before these proteases are encountered.

Like the effect of N-linked glycosylation on the overall final structure of other proteins (Machamer and Rose, 1988) it could also help the scFv in folding or protecting its linker peptide by steric effects from cleavage by proteases (Dullis *et al.*, 1982; Elbein, 1987). This modification did not improve the expression deficit of the secretory scFv. The amount of scFv-S expressed was too low to determine unambiguously whether it was indeed glycosylated. However the ER retained control constructs ($scFv^{LNC}$-SK and $scFv^{LNS}$-SK) were glycosylated indicating that the recognition sequence should also be accessible in the scFv-S molecules. The glycosylation status of both $scFv^{LNC}$-SK and $scFv^{LNS}$-SK was only partial, probably as a result of the context of the recognition site (Machamer and Rose, 1988; Shakin-Eshleman *et al.*, 1996). The fact that the glycosylated fraction is sensitive to endo H indicates that these molecules are present in the ER and/or cis-Golgi compartment (Pelham, 1988), which is in agreement with the location of the KDEL receptor (Semenza *et al.*, 1990; Lewis and Pelham, 1990). These results confirm the observations obtained with immunogold labeling (Artsaenko *et al.*, 1995; Schouten *et al.*, 1997). The fact that the accumulation cannot be increased is in contrast to what has been found for a different scFv molecule (Jost *et al.*, 1994).

The cysteine in the 202' derived linker could play a role in free thiol-mediated quality control of protein assembly (Fra *et al.*, 1993; Hammond and Helenius, 1994). The Cys to Ser mutation proved that the 65kDa product represents a dimer consisting of two scFv molecules connected by the cysteines in the peptide linker (see also: Chapter 5). The fact that we do not observe full dimerization could be caused by the acidic residue preceding the cysteine in the linker (Reddy *et al.*, 1996). Therefore this residue could be of particular relevance in relation to quality control mechanisms. In agreement with this is the twofold higher accumulation in the scFv-SK constructs upon replacement of the cysteine. However this

modification had no beneficial effect on the accumulation of secretory scFv (scFv-S), both intra- and extracellularly.
Despite the change of some of the apparent drawbacks in the amino acid sequence of the 202' derived linker we never observed scFv secretion to a level found by Firek *et al.*, (1994). Since they used a similar linker peptide these results might reflect differences in stability caused by the primary amino acid sequence of the variable domains.
Surprising results were obtained with the $(Gly_4Ser)_3$ linker (Huston *et al.*, 1988). This linker peptide has also been used in scFv antibodies expressed *in planta* (Tavladoraki *et al.*, 1993, Fiedler *et al.*, 1995 , Artsaenko *et al.*, 1995; Bruyns *et al.*, 1996). In these reports different architectures of the scFv have been employed. Therefore both $scFv^{L(gs)H}$ and $scFv^{H(gs)L}$ were created (Fig. 1B). These constructs showed the same expression results as the 202' linker constructs if the anti c-myc tag antibody was used for detection. However, using the anti-Fv antiserum it was found that the intracellular accumulation of the secretory scFv was increased, especially for the V_L-V_H architecture. The molecular weight of the expressed product was about 2 kDa smaller. This showed that the c-myc tag was removed since these molecules could not be detected with the anti c-myc tag antibody.
The increased intracellular accumulation of scFv with the $(Gly_4Ser)_3$ linker, did not result in a concomitant improvement of secretion. The level of secretion was similar to that found for the 202' derived linker constructs. Experiments with full size antibody constructs (unpublished results) indicate that the protoplasts are export competent since large amounts of antibodies are found in the medium. Apparently the major part of the scFv molecules is retained in the cell.
The $(Gly_4Ser)_3$ linker greatly improves the scFv stability, probably by decreasing proteolysis in the linker region. It thereby prevents the Fv regions to dissociate. However the removal of the c-myc tag indicates that the molecule still encounters proteases. Although we can exclude KEX-like proteases, it is unclear which proteases are responsible for this cleavage.
As found for other scFv molecules the domain order influences protein yield and binding activity (Dorai *et al.*, 1994, Tsumoto *et al.*, 1994). The same was found for the 21C5 scFv with the $(Gly_4Ser)_3$ linker. The $scFv^{H(gs)L}$ constructs was expressed less efficiently than the construct with reversed configuration ($scFv^{L(gs)H}$). Furthermore, the $scFv^{H(gs)L}$ constructs had a decreased affinity for the antigen or were more difficult to detect as a result of steric effects.
The presence of peptide sequences from the C_H1 region of the heavy chain in a scFv construct might influence its secretion as well as its stability. It has been shown that heavy chains were retained in the ER in the absence of the light chains. The C_H1 domain is critical for this retention (Hendershot *et al.*, 1987). In a scFv all residues on the former variable/constant domain interface become solvent exposed. On this interface the frequency of exposed hydrophobic residues is much higher than in the rest of the Fv fragment (Nieba *et al.*, 1996). In our scFv 21C5 we left five C_H1 residues at the C-terminus for cloning purposes. It follows that these residues could play an ambiguous role concerning the stability of the scFv. To study this feature these five amino acids were removed. However, the results obtained showed that this deletion was to no avail.
Since secretion of full size antibodies and Fab fragments in plants is generally more successful, scFv constructs were made which could assemble into more full size antibody-like molecules. There are many options to dimerize scFvs (Hoogenboom, 1997). We chose the IgM C_H4 domain and the camel IgG_2 long hinge and IgG_3 short hinge. It was demonstrated previously that IgG antibodies could multimerize when the IgM C_H4 domain

was added (Smith and Morrison, 1994). The use of the camel hinges for dimerization was a new approach. In camel the long and short hinges replace the C_H1 domain. These hinges are of interest for multimerization purposes since they are normally stable in antibody molecules which lack light chains (Hamers-Casterman, *et al.*, 1993) and do not arrest secretion by interaction with Bip, like the C_H1 domain in murine IgG antibodies does (Hendershot *et al.*, 1987). A combination of the two added to an scFv ($scFv^{L(gs)H}$-LH-C_H4-S fragments) may, in theory, not only form dimers but also multimeric forms of these dimers. About 50% of the $scFv^{L(gs)H}$-LH-S, $scFv^{L(gs)H}$-LH-SK and $scFv^{L(gs)H}$-C_H4-S fragments occurred as a dimer. Although in stable transformants, compared to $scFv^{L(gs)H}$-S, slightly better $scFv^{L(gs)H}$-LH-S expression was obtained, degradation still occurred and expression levels were low. Relatively high $scFv^{L(gs)H}$-C_H4-S protein levels (estimated at 0.2% of the total soluble protein) were obtained without detection of significant degradation products. However, in contrast to what was expected, both monomeric and dimeric fragments were mainly detected intracellularly. Remarkably, when both domains were added ($scFv^{L(gs)H}$-LH-C_H4-S) secretion was significantly improved. Since both monomers and dimers are secreted, intracellular transport competence and protein stability is not provided through dimer formation, as was found for IgM antibodies in animal cells (Alberini *et al.*, 1990).
The transient expression assays suggests that even polymeric $scFv^{L(gs)H}$-LH-C_H4-S fragments can be formed, although at a low percentage. In stable transformants however no polymeric fragments could be detected. The structural orientation of the cysteines may be such that they are preferentially involved in dimerization or the chaperones present may not be able to assemble $scFv^{L(gs)H}$-LH-C_H4-S fragments into polymers as efficiently as was found for chimeric IgG_3-C_H4 molecules in murine myeloma cells (Smith and Morrison, 1994).
The predictability of these transient expression results for the scFv behaviour in transgenic plants is unclear. Transient expression assays with $scFv^{L(gs)H}$-LH-S were promising and no intracellular degradation was visible. However, when stable transformants were analyzed one third expressed this fragment at a low protein level, whereas all of the $scFv^{L(gs)H}$-SK transformants analyzed showed a relative high protein expression. Furthermore, $scFv^{L(gs)H}$-LH-S protein degradation was visible in all screened transformants.
The analysis of the different scFv constructs allows us to follow part of the fate of scFv molecule in the secretory pathway. Previously, it was demonstrated that the $scFv^{RSC}$-SK was translocated into the ER (Schouten *et al.*, 1997). The deglycosylation experiments using the glycosylated scFv fragments confirm this. This is direct proof that the signal peptide exerts its function properly, like it did with the full size light and heavy chains (van Engelen *et al.*, 1994). The general consensus is that the immunoglobulin molecules are retained in the ER by interactions with GRP78 (BiP), GRP94 and calnexin (Bole *et al.*, 1986; Melnick *et al.*, 1994; Degen and Williams, 1991) until they are properly processed and have attained their properly folded and assembled structure. Homologues for these accessory proteins have been found in plants (Fontes *et al.*, 1991; Walther-Larsen *et al.*, 1993; Huang *et al.*, 1993) and it can therefore be assumed that the same mechanisms pertain in plant cells, although interactions with BiP are unlikely since scFvs lack the C_H1 domain (Hendershot *et al.*, 1987). From the ER onwards the pathway that scFv molecules follow diverges between the scFv-SK and scFv-S versions. The scFv-SK molecules are recycled from the cis-Golgi back into the ER by their association with the KDEL receptor (Pelham 1992). The scFv-S protein is expected to leave the cell. However, despite its stability in the protoplast culture medium, it is only found in low amounts. Therefore, degradation may occur beyond the cis-Golgi. In mammalian cells some form of quality control mechanisms operates in post ER

compartments (Hammond and Helenius, 1995). Proteolytic processing of polypeptides occurs in the trans-Golgi compartment or later (Sambamurti *et al.*, 1992; Lahiri, 1994; Deng *et al.*, 1995; Cartwright and Higgins, 1996; Wilnow *et al.*, 1996), indicating that specific proteases are located there

Quality control mechanisms, present in the ER may be responsible for the arrest in intracellular transport. The same phenomenon has been reported for the behaviour of a single domain antibody in plants (Benvenuto *et al.*, 1991). In animal cells some scFvs (Marasco *et al.*, 1993; Jost *et al.*, 1994) and even individual light chains (Wu *et al.*, 1983; Dul and Argon, 1990) show this lack of secretion. Based on our results for the scFvs with modified linker it is not possible to discriminate between impairment in exit from the ER or a later intracellular compartment.

The results obtained with the secreting scFv fragment, $scFv^{L(gs)H}$-LH-C_H4-S, give a more detailed picture with respect to quality control mechanisms. The deglycosylation experiments demonstrate that these fragments have not passed the cis-Golgi, suggesting that a rate limiting step mediates the exit from the ER or cis-Golgi. This is probably not caused by the intermolecular disulfide bridge formation since both monomeric and dimeric fragments are secreted equally efficient into the medium. Due to the rigid rod shaped structure of the long hinge (Hamers-Casterman *et al.*, 1993) and the positions of the cysteine residues it seems unlikely that intra molecular disulfide bridges within the long hinge and/or C_H4 are present in the monomeric fragments. Furthermore, incubation with 2ME does not impair secretion, suggesting that the free cysteines are not involved in quality control of $scFv^{L(gs)H}$-LH-C_H4-S. 2ME displays only positive effects on the secretion of IgM intermediates that are retained by disulfide interchange reactions involving the C_H4 Cys575 reactions (Valetti and Sitia, 1994). Since DTT is more effective in reducing intra- and intermolecular disulfide bridges (Valetti and Sitia, 1994), the overall structure of $scFv^{L(gs)H}$-LH-C_H4-S is disrupted to a level that chaperones, involved in a correct folding, retain the fragment from being secreted. Additionally, this may indicate that, under natural conditions, the $scFv^{L(gs)H}$-LH-C_H4-S protein is correctly folded.

Treatment with tunicamycin did result in a higher $scFv^{L(gs)H}$-LH-C_H4-S secretion level and lower intracellular accumulation, suggesting that the quality control mechanism with respect to glycosylation is the rate limiting step for secretion. Since tunicamycin prevents glycoproteins, and thus the C_H4 domain, from being glycosylated, $scFv^{L(gs)H}$-LH-C_H4-S will not be bound to the chaperone calnexin (Hammond *et al.*, 1994). Normally, calnexin will retain only those glycoproteins which still carry glucose residues and, therefore, have not yet been properly trimmed. Noteworthy in this respect is that an IgM antibody which was designed to be secreted in tobacco was also found only intracellularly (Düring *et al.*, 1990). In all, this may suggest that the maturation of glycosylation of antibodies and antibody fragments in the ER in some cases proceeds relatively inefficient. Although the N-linked glycosylation process of proteins in plants is similar to that in mammals (Jones and Robinson, 1989), there are differences in added terminal residues (Sturm *et al.*, 1987). Hein *et al.* (1991) reported that an IgG antibody synthesized by plants differed from the same antibody synthesized by hybridoma cells with respect to glycosylation. This may explain why the secreted $scFv^{L(gs)H}$-LH-C_H4-S was not sensitive to GlycoF treatment. Glycosylation of the C_H4 domain may also differ, resulting in a modification that is GlycoF resistant, as a consequence of the addition of a core α1-3 fucose (Tretter *et al.*, 1991). This kind of modification can be found frequently in plant glycoproteins (Sturm *et al.*, 1987). The nascent C_H4 domain will rapidly begin with the formation of the proper domain structure the moment

it is translocated into the ER, which may make it difficult for modifying enzymes, not found in mammalian cells, to process the glycans. A chaperone involved in the quality control mechanism may therefore, similar to calnexin, prevent the partially processed protein from exiting the ER.

Our results strongly suggest that the architecture and linker introduced in the scFv are not the main reason for a low secretion level. Apparently residues in the frameworks or complementarity-determining regions are responsible for this. In this respect it is interesting to note that in stable transformants we found relatively high amounts of non-degraded scFv, which was derived from a different antibody (unpublished results). Therefore, apart from the design, scFv secretion in plant cells probably depends also upon the primary amino acid sequence of the domains which influence the inherent stability of the combination of light and heavy chain variable domains, both of which can belong to considerable number of subtypes (Kabat *et al.*, 1991). In full size antibodies probably the contributions of the variable regions to the total stability are low as compared to those of the constant domains (Glockshuber *et al.*, 1990) and therefore do not pose a strong selection against the use of certain combinations of subtypes. In a single-chain format and in an heterologous system however certain combinations of variable regions might highlight their less favourable association by a lowered expression and secretion level. The addition of extra constant domains C-terminally may therefore render the overall structural stability less dependent on the contributions of the variable domains and therefore may be beneficial for the secretion of otherwise poorly secreted scFvs.

CHAPTER 7

Summary and Concluding Remarks

A major challenge with respect to the expression of antibodies or antibody fragments in plants is to obtain large amounts of correctly folded and functional proteins at the proper subcellular location. In this thesis it is demonstrated that antibodies and single-chain Fv fragments (scFvs) can be efficiently expressed in plants, targeted to the proper subcellular location and the subcellular location influences expression levels and functionality.

Coordinate expression of full size antibody coding genes.

The most important condition for full size antibodies is that two genes must be expressed equally efficient to guarantee optimal antibody expression levels. In Chapter 2 an efficient transformation vector is described for integration of a single T-DNA, carrying both the heavy and light chain encoding genes, into the plant genome. Controlled by the CaMV 35S and the TR2' promoter both genes were efficiently expressed simultaneously, giving rise to a large proportion of transformants expressing a complete antibody at a relatively high level in roots and leafs (Wilmink *et al.*, 1998). This approach avoids the time consuming method of crossing light chain expressing transgenics with heavy chain expressing transgenics (Hiatt *et al.*, 1989) or the unreliable method of co-transformation, which suffers from a large proportion of low antibody expressors (De Neve *et al.*, 1993). Furthermore, segregation in the progeny is avoided.

The secreted antibodies were to some extent sensitive to proteolytic processing, resulting in functional $F(ab')_2$-like molecules. These results and the observations by others (Hein *et al.*, 1991; Voss *et* al., 1995; de Wilde *et* al., 1996) illustrate that antibodies of 150 kDa can pass the cell wall and accumulate in the apoplastic space. De Wilde *et al.* (1998) also demonstrated that antibodies can be transported apoplasticly through the plant.

In this thesis cytosolic expression of complete antibodies was not studied. In animal cells the assembly of full size antibodies has been described (Biocca *et al.*, 1990). In plants, however, cytosolic expression resulted only in very low levels of unassembled light and heavy chains (Hiatt *et al.*, 1989). Chaperones present in the cytosol may promote the correct folding of the individual peptide chains, but the reducing environment probably prevents the assembly into complete antibodies through disulfide bridge formation.

Subcellular targeting of single-chain antibodies in plant cells.

The unique characteristic of scFvs is that they can be modified in a relatively simple way to obtain optimal stability and functionality at various interesting subcellular locations in plants (Fig. 1). When carrying the signal peptide for translocation into the ER an anti-cutinase scFv can be secreted, although often poorly. It was efficiently retained to high levels of functional protein in the endoplasmic reticulum (ER) by adding the ER translocation signal and the four amino acid extension, Lys-Asp-Glu-Leu (or KDEL). Most intriguing, cytosolic expression of the scFv failed unless the KDEL extension was added C-terminally (Chapter 3). Immunoelectron microscopy and molecular markers confirmed that the cytosolic scFv with KDEL extension was located in the cytosol and not 'mistranslocated' to another subcellular compartment. Although the mode of action remains elusive, addition of this four amino acid extension, or a slightly modified one (KDEI), also improved cytosolic expression of several other scFv fragments (Chapter 4). Protein degradation commencing at the C-terminus may be prevented or the extension may sterically protect another part of the scFv fragment, which is susceptible to proteolysis.

Functional scFvs in the reducing cytosolic environment.

Interestingly, further characterization of stable transformants revealed that the cytosolic scFv was present in an oxidized and functional state, like the ER retained fragment. The intramolecular disulfide bridges in the variable domains were present. Furthermore, cysteine residues present in the linker could, partially, form intermolecular disulfide bonds, resulting in scFv dimers. However, transient expression led to a non-functional cytosolic scFv, which lacked disulfide bridges (Chapter 5). This illustrates that the disulfide bridges in the variable domain are crucial for functionality (Fig. 2).

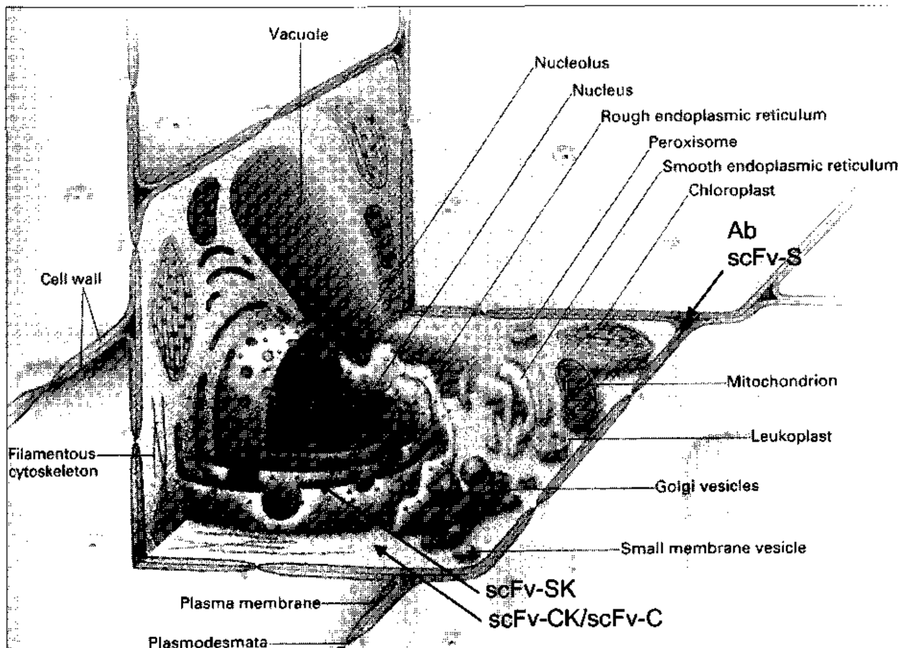

Fig. 1. Subcellular targeting of functional antibodies and scFv fragments in plant cells (Lodish *et al.*, 1995). Secreted complete antibodies (Ab) and scFvs (scFv-S) can traverse the cell wall. ScFv can be intracellularly expressed in the endoplasmic reticulum, by adding the ER retention signal KDEL (scFv-SK) to the secretory scFv, or in the cytosol by omitting the ER translocation signal (scFv-C/scFv-CK).

Disulfide bridge formation in the cytosol is remarkable. The cytosol and the secretory pathway of eukaryotic cells are two environments of grossly different thiol-disulfide redox states. In the oxidizing environment of the secretory pathway the formation of disulfide bridges is an important step in post translational processing. This is essential for folding, oligomerization and sorting of the assembled protein among different pathways.

In contrast, in the reducing environment of the cytosol, formation of disulfide bonds is considered to be rare. The cysteine residues present in cytosolic proteins are often being used to bind ligands in Cys(S)-X bonds, where X= Fe, Zn, S, C or Cu. When present, disulfide bridges are often associated with an inactivated protein. However, evidence about

the *in vivo* redox state of cytosolic proteins is scarce and most has been deduced indirectly from *in vitro* experiments. Like in animal cells, the redox potential in the cytosol of plant cells is not static, but there is little information between which values it varies. *In vitro* experiments indicated that scFvs could fold efficiently at mildly reducing conditions (Ryabova *et al.*, 1997). Apparently, the intrinsic properties of the protein determine the eventual redox state of the protein at a given redox potential in the cytosol. The fact that the transiently expressed scFv is present in a reduced and non-functional state can therefore probably be explained by the physiological conditions of the tobacco protoplasts. When put under oxidative stress conditions, the cytosol of plant cells becomes a more reducing environment, thus preventing scFvs from becoming oxidized.

In conclusion, unlike the cytosol of bacteria, the plant cytosol may not be such a hostile environment for the expression of functional scFv. Formation of the proper intramolecular disulfide bonds to obtain functional scFvs is possible in stable transformants. This makes scFv fragments ideal to modulate protein activity in the plant cytosol and, as a consequence, to alter existing or design new metabolic pathways and to obtain resistance against pathogens. This approach is supported by the successful results of Tavadoraki *et al.* (1993) and Owen *et al.* (1992).

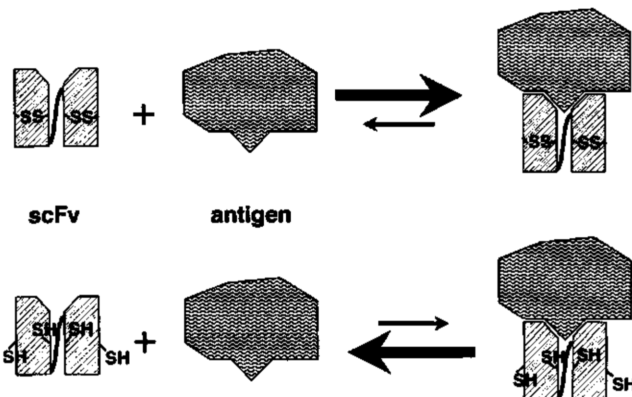

Fig. 2. Intramolecular disulfide bridges within the variable domains of the scFv fragment are crucial for antigen binding capabilities.

ScFv secretion by plant cells.

Secretion of scFv fragments proved to be more difficult than anticipated (Chapter 3). However, low expression could not be related to a poor transcription or translation. For the ER retained scFv expression levels of 1% were reached. Apparently, the scFv is susceptible to proteolytic degradation further down the secretory pathway. Since in the literature reported scFvs differed with respect to variable domains, architecture, linker peptide and C-terminal residues it was difficult to determine which of the individual factors determine low scFv secretion.

To elucidate the causes of this phenomenon a systematic study was set up to analyze whether scFv expression and secretion could be improved without changing the variable domains and thus the specificity. Several modifications in the linker peptide were introduced to reduce possible susceptibility to certain proteases. This was to no avail. However,

exchanging the 202' linker by the other frequently used $(Gly_4Ser)_3$ *linker and maintaining the* V_L-V_H architecture of the poorly secreting 21C5 scFv resulted in an elevated intracellular scFv expression level when expressed transiently in tobacco protoplasts comparable to the ER retained scFv. The expression levels were comparable to those of the ER retained scFv. Although functional, this protein was still badly secreted. Apparently, secretion is arrested somewhere in the secretory pathway, probably by a protein quality control mechanism. No stable transformants were found expressing this scFv fragment at detectable levels. This may emphasize that, as suggested before (Glockshuber *et al.*, 1990), the contribution of the variable domains to stabilize protein folds in an scFv are rather low, resulting in a structure in which proteolytic sensitive sites still can be reached.

In an effort to reduce the overall contribution of the variable domains to stability extra constant domains were added C-terminally. These domains were also expected to cause dimerization through disulfide bridge formation (Fig. 3). The selected domains, the IgM C_H4 domain and the camel IgG_2 long hinge, have interesting properties. The C_H4 domain is involved in pentamer formation of IgM antibodies and has already been used successfully to

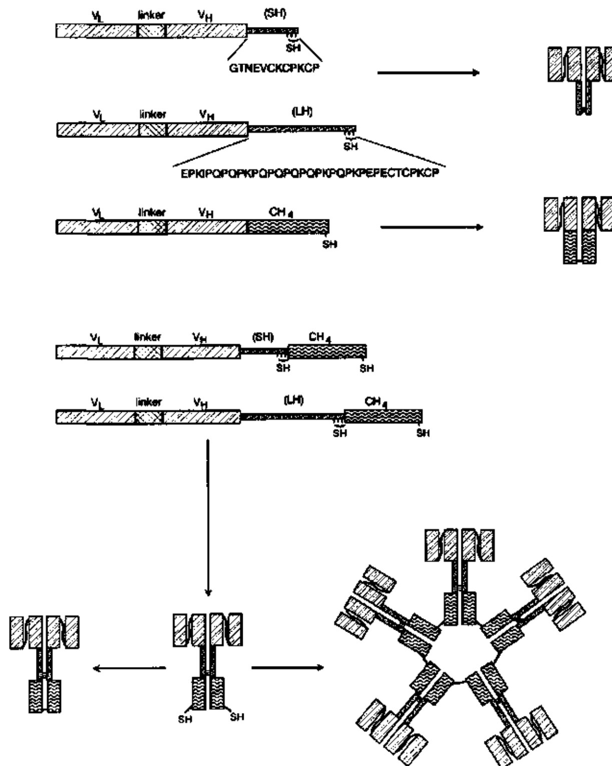

Fig. 3. Construction of scFv fragments with extra domains for the purpose of mulimerization through disulfide bridge formation. Addition of the IgM C_H4 (C_H4) domain and the camel IgG_2 long hinge (LH) and IgG_3 short hinge (SH) leads to dimerization. Addition of both the LH and C_H4 domain may lead to multimeric IgM like structures.

multimerize IgG antibodies (Smith and Morrison, 1994). The camel IgG_2 long hinge and IgG_3 short hinge are rod shaped like domains which substitute the C_H1 domain normally found in murine IgG antibodies. In addition, these camel IgG_2 and IgG_3 antibodies lack light chains. Consequently, the long and short hinges do not depend on an interaction with the C_L domain for their stability. Adding these hinges to an scFv fragment would confront them with a similar situation. Both in transient expression assays and in stable transformants the addition of an IgM C_H4 domain did increase intracellular expression levels. Again, no secretion was observed. Remarkably, when both the camel IgG_2 long hinge and IgM C_H4 domain were added, the scFv fragment was partially secreted both as monomer and dimer. In this case, the intracellular accumulation is probably caused by an inefficient glycosylation of the C_H4 domain, which caused a prolonged stay of the fragment in the ER.

Transient expression suggested that scFv fragments carrying a long hinge combined with a CH_4 domain were, next to dimers, capable of forming trimers and tertramers. However, IgM-like multimers, as depicted in Fig. 3, were not observed. In addition, the trimers and tetramers were not found in stable transformants. Further optimization may eventually lead to an efficient assembly into IgM-like structures.

The individual domains are capable of folding separately into stable structures, enabling the design and expression of many exotic antibody constructs (Hoogenboom, 1997). However, our results indicate that antibody domains have their limitations, at least in plants, and one should be careful to consider domains as individual building blocks, which can be assembled into antibody fragments with any predicted structure.

In all, scFvs can be constructed which remain stable in the secretory pathway and become secreted more efficiently. By removing the glycosylation signal in the C_H4 domain scFv-LH-C_H4 fragments may be obtained which are secreted even more efficiently. New scFv constructs may also solve the problem of low scFv secretion, like bivalent scFv antibodies (Fig. 4). In these antibody fragments, an additional peptide linker covalently connects two scFvs. Both scFv fragments do not necessarily have the same specificity and different scFv can be linked. The first results show that these constructs can be expressed in plants (unpublished data).

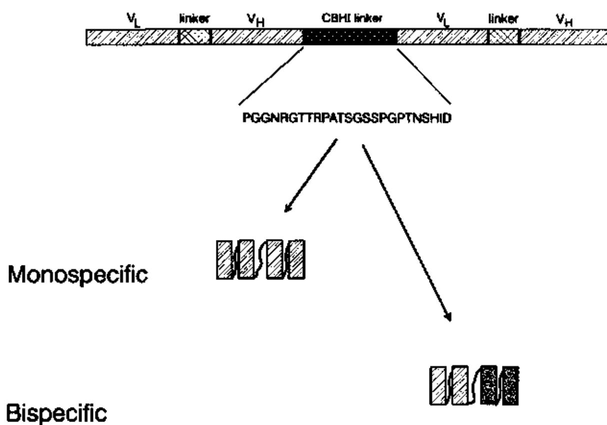

Fig. 4. Mono- and bispecific bivalent scFvs

Future prospects of the plantibody technology.

This thesis demonstrates that antibodies are flexible molecules, which facilitate obtaining transgenics expressing antigen-binding domains at high levels and at various subcellular locations. The first examples of modified plant traits and resistance against viruses show that plantibodies have an enormous potential. Once suitable antibodies are available, a rational approach can be devised to express the antibody at the appropriate subcellular location in the plant. Recently, promising target proteins in sedentary plant parasitic nematodes have been isolated and characterized (Smant *et al.*, 1998). Resistance not only to viruses but also to more complex organisms like nematodes, fungi and even insects through plantibody technology seems within reach.

References

Abate, C., Patel, L., Rauscher III, F.J. and Curran, T. (1990). Redox regulation of Fos and Jun DNA-binding activity in vitro. *Science* 249, 1157-1161.

Alberini, C.M., Bet, P., Milstein, C. and Sitia, R. (1990). Secretion of immunoglubulin M assembly intermediates in the presence of reducing agents. *Nature* **347**, 485-487.

Allen, S., Naim, H.Y. and Bulleid, N.J. (1995). Intracellular folding of tissue-type plasminogen activator - Effects of disulfide bond formation on N-linked glycosylation and secretion. *Journal of Biological Chemistry* **270**, 4797-4804.

Alosi, M.C., Melroy, D.L. and Park, R.B. (1988). The regulation of gelation of phloem exudate from *Cucurbita* fruit by dilution, glutathione, and glutathione reductase. *Plant Physiollogy* **86**, 1089-1094.

Alscher, R.G. (1989). Biosynthesis and antioxidant function of glutathione in plants. *Physiologia Plantarum* **77**, 457-464.

Amzel, M. and Poljak, R.J. (1979). Three-dimensional structure of immunoglobulins. *Annual Review of Biochemistry* **48**, 961-997.

An, G. (1986). Development of plant promoter expression vectors and their use for analysis of differential activity of nopaline synthase promoter in transformed tobacco cells. *Plant Physiology* **81**, 86-91.

Anand, N.N., Mandal, S., MacKenzie, C.R., Sadowska, J., Sigurskjold, B., Young N.M., Bundle, D.R. and Narang, S.A. (1991). Bacterial expression and secretion of various sngle-chain Fv genes encoding proteins specific for a Salmonella Sertype B O-antigen. *Journal of Biological Chemistry* **266**, 21874-21879.

Anderson, L.E., Li, D., Prakash, N. and Stevens, F.J. (1995). Identification of potential redox-sensitive cysteines in cytosolic forms of fructosebisphosphatase and glyceraldehyde-3-phosphate dehydrogenase. *Planta* **196**, 118-124.

Artsaenko, O., Peisker, M.. Zurnieden, U., Fiedler, U., Weiler, E.W., Muntz, K. and Conrad, U. (1995). Expression of a single-chain Fv antibody against abscisic acid creates a wilty phenotype in transgenic tobacco. *Plant Journal* **8**, 745-750.

Assaad, F.F., Tucker, K.L. and Signer, E.R. (1993). Epigenetic repeat-induced gene silencing (RIGS) in *Arabidopsis*. *Plant Molecular Biology* **22**, 1067-1085.

Benfey, P.N., Ren, L. and Chua, N.-H. (1989). The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. *EMBO Journal* **8**, 2195-2202.

Benvenuto, E., Ordàs, R.J., Tavazza, R., Ancora, G., Biocca, S., Cattaneo, A. and Galeffi, P. (1991). 'Phytoantibodies': a general vector for the expression of immunoglobulin domains in transgenic plants. *Plant Molecular Biology* **17**, 865-874.

Bergman, L.W. and Kuehl, W.M. (1979). Formation of an intrachain disulfide bond on nascent immunoglobulin light chains. *Journal of Biological Chemistry* **254**, 8869-8876.

Bibi, E. and Laskov, R. (1990). Selection and application of antibodies modifying the function of β-lactamase. *Biochimica et Biophysica Acta* **1035**, 237-241.

Biocca, S., Neuberger, M.S. and Cattaneo, A. (1990). Expression and targeting of intracellular antibodies in mammalian cells. *EMBO Journal* **9**, 101-108.

Biocca, S., Pierandreiamaldi, P. and Cattaneo, A. (1993). Intracellular expression of anti-P2L(Ras) single chain Fv fragments inhibits meiotic maturation of *Xenopus* oocytes. *Biochemical and Biophysical Research Communications* **197**, 422-427.

Biocca, S., Pierandreiamaldi, P., Campioni, N. and Cattaneo A. (1994). Intracellular immunization with cytosolic recombinant antibodies. *Bio/technology* **12**, 396-399.

Biocca, S., Ruberti, F., Tafani, R., Pierandreiamaldi, P. and Cattaneo, A. (1995). Redox state of single chain Fv fragments targeted to the endoplasmic reticulum, cytosol and mitochondria. *Bio/technology* **13**, 1110-1115.

Biocca, S. and Cattaneo, A. (1995). Intracellular immunization: Antibody targeting to subcellular compartments. *Trends in Cell Biolology* **5**, 248-252.

Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufman, B.M., Lee, S.- M., Lee, T., Pope, S.H., Riordan, G.S. and Whitlow, M. (1988). Single-chain antigen-binding proteins. *Science* **242**, 423-426.

Bird, R.E. and Walker, B.W. (1991). Single chain antibody variable regions. *Trends in Biotechnology* **9**, 132-137.

Bole, D.G., Hendershot, L.M. and Kearny, J.F. (1986). Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *Journal of Cell Biology* **102**, 1558-1566.

Bosch, D., Smal, J. and Krebbers, E. (1994). A trout hormone is expressed, correctly folded and partially glycosylated in the leaves but not the seeds of transgenic plants. *Transgenic Research* **3**, 304-310.

Braakman, I., Helenius, J. and Helenius, A. (1992). Role of ATP and disulfide bonds during protein folding in the endoplasmic reticulum. *Nature* **356,** 260-262.

Breyne, P., Gheysen G., Jacobs A., Van Montagu M. and Depicker A. (1992). Effect of T-DNA configuration on transgene expression. *Molecular and General Genetics* **235**, 389-396.

Bruyns, A.M., Dejaeger, G., Deneve, M., Dewilde, C., Vanmontagu, M. and Depicker, A. (1996). Bacterial and plant-produced scFv proteins have similar antigen- binding properties. *FEBS Letters* **386**, 5-10.

Carlson, J.R. (1988). A new means of inducibly inactivating a cellular protein. *Molecular Cell Biology* **8**,2638-2646.

Cartwright, I.J. and Higgins, J.A. (1996). Intracellular degradation in the regulation of secretion of apolipoprotein B-100 by rabit hepatocytes. *Biochemical Journal* **314**, 977-984

Chanat, E., Weiss, U., Huttner, W.B. and Tooze, S.A. (1993). Reduction of the disulfide bond of chromogranin B (secretogranin I) in the trans-Golgi network causes its missorting to the constitutive secretory pathway. *EMBO Journal* **12,** 2159-2168.

Chaudhary, V.K., Batra, J.K., Gallo, M.G., Willingham, M.C., FitzGerald, D.J. and Pastan, I. (1990). A rapid method of cloning functional variable-region antibody genes in *Escherichia coli* as single-chain immunotoxins. *Proceedings of the National Academy of Sciences USA* **87**, 1066-1070.

Chen, W., Helenius, J., Braakman, I. and Helenius, A. (1995). Cotranslational folding and calnexin binding during glycoprotein synthesis. *Proceedings of the National Academy of Sciences USA* **92,** 6229-6233.

Choudary, A.D., Lamb, C.J. and Dixon, R.A. (1990). Stress responses in alfalfa (*Medicago sativa* L.) VI. Responsiveness of chalcone synthase induction to fungal elicitor or glutathione in electroporated protoplasts. *Plant Physiol.* **94,** 1802-1807.

Church, G.M. and Gilbert, W. (1984). Genomic sequencing.*Proceedings of the National Academy of Sciences USA* **81**, 1991-1995.

Cséke, C. and Buchanan, B.B. (1986). Regulation of the formation and utilization of photosynthate in leaves. *Biochimica et Biophysica Acta* **853,** 43-63.

Czichi, U. and Lennarz, W.J. (1977). Localization of the enzyme system for glycosylation of proteins via the lipid-linked pathway in rough endoplasmic reticulum. *Journal of Biological Chemistry* **252**, 7901-7904.

Dahmus, M.E., Laybourn, P. and Borrebaeck, C.A.K. (1988). Production of monoclonal antibody against electrophoretic purified RNA polymerase II subunits using *in vitro* immunization. *Molecular Immunology* **25,** 997-1003.

Dangl, J.L. Wensel, T.G., Morrison, S.L., Stryer,L., Herzenberg, L.A. and Oi, V.T. (1988). Segmental flexibility and complement fixation of genetically engineered chimeric human, rabbit and mouse antibodies. *EMBO Journal* **7,** 1989-1994.

Dean, C., Favreau, M., Tamaki, S., Bond-Nutter, D., Dunsmuir P. and Bedbrook J. (1988). Expression of tandem gene fusions in transgenic tobacco plants. *Nucleic Acids Research* **16**, 7601-7617.

de Boer, J.M., Smant, G., Goverse, A., Davis, E.L., Overmars, H.A., Pomp H., Van Gent-Pelzer, M., Zilverentant, J.F., Stokkermans, J.P.W.G., Hussey, R.S., Gommers, F.J., Bakker, J. and Schots, A. (1996). Secretory granule proteins from the subventral esophageal glands of the potato cyst nematode identified by monoclonal antibodies to a protein fraction from second-stage juveniles. *Molecular Plant-Microbe Interactions* **9**, 39-46.

Degen, E., Williams, D.B. (1991). Participation of a novel 88-kD protein in the biogenesis of murine class I histocompatibility molecules. *Journal of Cell Biology* **112**, 1099-1115

Denecke, J., Gosselé, V., Botterman, J. and Cornelissen, M. (1989). Quantitative analysis of transiently expressed genes in plant cells. *Methods in Molecular Cell Biology* **1**, 19-27.

Denecke, J., De Ryke, R. and Botterman, J. (1992). Plant and mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope. *EMBO Journal* **11**, 2345-2355.

De Neve, M., Deloose, M., Jacobs, A., Vanhoudt, H., Kaluza, B., Weidle, U., van Montagu M. and Depicker, A. (1993). Assembly of an antibody and its derived antibody fragment in *Nicotiana* and *Arabidopsis*. *Transgenic Research* **2**, 227-237.

Deng, J., Rudick, V. and Dory, L. (1995). Lysosomal degradation and sorting of apolipoprotein E in macrophages. *Journal of Lipid Research* **36**, 2129-2140.

Desplancq, D., King, D.J., Lawson, A.D.G. and Mountain, A. (1994). Multimerization behaviour of single chain Fv variants for the tumour-binding antibody B72.3. *Protein Engineering* **7**, 1027-1033.

Devereux, J., Haerbeli, P. and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* **12**, 387-395.

De Vries, S., Hoge, H. and Bisseling, T. (1991). Isolation of total and polysomal RNA from plant tissues. In. Gelvin, B., Schilperoort, R.A. and Verma, D.P.S. (eds.) *Plant Molecular Biology Manual*, pp. B6/1-B6/13. Kluwer Academic Publishers, Dordrecht.

de Vries, A.A.F., Raamsman, M.J.B., van Dijk, H.A., Horzinek, M.C. and Rottier, P.J.M. (1995). The small envelope glycoprotein (G_s). of equine arteritis virus folds into three distinct monomers and a disulfide-linked dimer. *Journal of Virology* **69**, 3441-3448.

De Wilde, C., De Neve, M., De Ryke, R., Bruins, A.-M., De Jaeger, G., Van Montagu, M., Depicker, A. and Engler, G. (1996). Intact antigen-binding MAK33 antibody Fab fragment accumulate in intracellular spaces of *Arabidopsis thaliana*. *Plant Science* **114**, 233-241.

De Wilde, C., De Ryke, R., Beeckman, T., De Neve, M., Van Montagu, M., Engler, G. and Depicker, A. (1998). Accumulation pattern of IgG antibodies and Fab fragments in transgenic *Arabidopsis thaliana* plants. *Plant and Cell Physiology* **39**, 639-646.

Dorai, H., Mccartney, J.E., Hudziak, R.M., Tai, M.S., Laminet, A.A., Houston, L.L., Huston, J.S. and Oppermann, H. (1994). *Mammalian cell expression of single-chain Fv (sFv) antibody proteins and* their C-terminal fusions with interleukin-2 and other effector domains. *Bio/technology* **12**, 890-897.

Duan, L.X., Bagasra, O., Laughlin, M.A., Oakes, J.W. and Pomerantz, R.J. (1994). Potent inhibition of human immunodeficiency virus type 1 replication by an intracellular anti-Rev single-chain antibody. *Proceedings of the National Academy of Sciences USA* **91**, 5075-5079.

Dul, J.L. and Argon, Y.A. (1990). A single amino acid substitution in the variable region of the light chain specifically blocks immunoglobulin secretion. *Proceedings of the National Academy of Sciences USA.* **87**, 8135-8139.

Dulis, B.H., Kloppel, T.M., Grey, H.M. and Kubo, R.T. (1982). Regulation of catabolism of IgM heavy chains in a B lymphoma cell line. *Journal of Biological Chemistry* **257**, 4369-4374.

Düring, K., Hippe, S., Kreuzaler, F. and Schell, J. (1990). Synthesis and self-assembly of a functional monoclonal antibody in transgenic *Nicotiana tabacum*. *Plant Molecular Biology* **15**, 281-293.

Elbein, A.D. (1987). Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. *Annual Review of Biochemistry* **56**, 497-534.

Fecker, L.F., Kaufmann, N., Commandeur, U., Commandeur, J., Koenig, R. and Burgermeister, W. (1997). Expression of single-chain antibody fragments (scFv) specific for beet necrotic yellow vein coat protein or 25 kDa protein in *Escherichia coli* and *Nicotiana benthamiana*. *Plant Molecular Biology* **32**, 979-986.

Fecker, L.F., Koenig, R. and Obermeier, C. (1997). *Nicotiana Benthamiana* plants expressing beet necrotic yellow vein virus (BNYVV) coat protein-specific scFv are partially protected against the establishment of the virus in the early stages of infection and its pathogenic effects in the late stages of infection. *Archives of Virology* **142**, 1857-1863.

Feinberg, A.P. and Fogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**, 6-13.

Fiedler, U. and Conrad, U. (1995). High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds. *Bio/technology* **13**, 1090-1093.

Firek, S., Draper, J., Owen, M.R.L., Gandecha, A., Cockburn, B. and Whitelam, G.C. (1993). Secretion of a functional single-chain Fv protein in transgenic tobacco plants and cell suspension cultures. *Plant Molecular Biology* **23**, 861-870.

Flipse, E., Huisman, J.G., de Vries, B.J., Bergervoet, J.E.M., Jacobsen, E. and Visser, R.G.F. (1994). Expression of a wild-type GBSS gene introduced into an amylose-free potato mutant by *Agrobacterium tumefaciens* and the inheritance of the inserts at the microsporic level. *Theoretical and Applied Genetics* **88**, 369-375.

Florack, D.E,A., Dirkse, W.G., Visser, B., Heidekamp, F. and Stiekema, W.J. (1994). Expression of biologically active hordothionins in tobacco. Effects of pre and pro-sequences at the amino and carboxyl termini of the hordothionin precursor on mature protein expression and sorting. *Plant Molecular Biology* **24**, 83-96.

Fontes, E.B.P., Shank, B.B., Wrobel, R.L., Moose, S.P., OBrian, G.R., Wurtzel, E.T. and Boston, R.S. (1991). Characterization of an immunglobulin binding protein homolog in the maize floury-2 endosperm mutant. *Plant Cell,* 483-496.

Fra, A.M., Fagioli, C., Finazzi, D., Sitia, R. and Albertini, C.M. (1993). Quality control of ER synthesized proteins: an exposed thiol group as a three-way switch mediating assembly, retention and degeneration. *EMBO Journal* **12**, 4755-4761.

Freedman, R.B. (1989). Protein disulphide isomerase. Multiple roles in the modification of nascent secretory proteins. *Cell* **57**, 1069-1072.

Freund, C., Honegger, A., Hunziker, P., Holak, T.A. and Plückthun, A. (1996). Folding nuclei of the scFv fragment of an antibody. *Biochemistry* **35**, 8457-8464.

Garaci, E., Palamara, A.T., Ciriolo, M.R., D'Agostini, C., Ab del-Latif, M.S., Aquaro, S., Lafivia, E. and Rotillio, G. (1997). Intracellular GSH content and HIV replication in human macrophages. *Journal of Leukocyte Biology* **62**, 54-59.

Gardner, A.M., Aviel, S. and Argon, Y. (1993). Rapid degradation of an unassembled immunoglobulin light chain is mediated by a serine protease and occurs in a pre-Golgi compartment. *Journal of Biological Chemistry* **268**, 25940-25947.

Gavel, Y. and Von-Heijne, G (1990). Sequence differences between glycosylated and non-glycosylated Asn-X-Thr-Ser acceptor sites: Implications for protein engineering. *Protein Engineering* **3**, 433-442.

Gething, M.-J. and Sambrook, J. (1992). Protein folding in the cell. *Nature* **355,** 33-44.

Gidoni, D., Bond-Nutter, D., Brosio, P., Jones, J., Bedbrook, J. and Dunsmuir P. (1988). Coordinated expression between two photosynthetic petunia genes in transgenic plants. *Molecular and General Genetics* **211**, 507-514.

Gilbert, H.F. (1990). Molecular and cellular aspects of thiol-disulfide exchange. *Advances in Enzymology* **63,** 69-172.

Glockshuber, R., Malia, M., Pfitzinger, I. and Plücktun, A. (1990). A comparison of strategies to stabilize immunoglobulin Fv-fragments. *Biochemistry* **29**, 1362-1367.

Glockshuber, R., Schmidt, T. and Plückthun, A. (1992). The disulphide bonds in antibody variable domains: Effects on stability, folding in vitro, and functional expression in *Escherichia coli. Biochemistry* **31,** 1270-1279.

Goto, Y. and Hamaguchi, K. (1979). The role of the intrachain disulfide bond in the conformation and stability of the constant fragment of the immunoglobulin light chain. *Journal of Biochem.* **86,** 1433-1441.

Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Bajyana Songa, E., Bendahman, N. and Hamers, R. (1993). Naturally occuring antibodies devoid of light chains. *Nature* **363**, 446-448.

Hammond, C. and Helenius, A. (1994). Quality control in the secretory pathway: Retention of a misfolded viral membrane glycoprotein involves cycling between the ER, intermediate compartment, and Golgi apparatus. *Journal of Cell Biology* **126**, 41-52.

Hammond, C. and Helenius, A. (1995). Quality control in the secretory pathway. *Current Opinion in Cell Biology* **7**, 523-529.

Hein, M.B., Tang, Y., McLeod, D.A., Janda, K.D. and Hiatt A. (1991). Evaluation of immunoglobulins from plant cells. *Bio/technolgy in Progress* **7**, 455-461.

Hendershot, L., Bole, D., Koehler, G. and Kearney, J.F. (1987). Assembly and secretion of heavy chains that do not associate posttranslationally with immunoglobulin heavy chain-binding protein. *Journal of Cell Biology* **104**, 761-767.

Herman, E.M., Tague, B.W., Hoffman, L.M., Kjemtrup, S.E. and Chrispeels, M.J. (1990). Retention of phytohemagglutinin with carboxyterminal tetrapeptide KDEL in the nuclear envelope and the endoplasmic reticulum. *Planta* **182**, 305-312.

Hérouart, D., van Montagu, M. and Inzé, D. (1993). Redox-activated expression of the cytosolic copper/zinc superoxide dismutase gene in *Nicotiana. Proceedings of the National Academy of Sciences USA* **90,** 3108-3112.

Hiatt, A., Cafferkey, R. and Bowdish, K. (1989). Production of antibodies in plants. *Nature* **342**, 76-78.

Hiatt, A. (1990). Antibodies produced in plants. *Nature* **344**, 469-470.

Holst, B., Bruun, A.W., Kielland-Brandt, M.C. and Winther, J.R. (1996). Competition between folding and glycosylation in the endoplasmic reticulum. *EMBO Journal* **15**, 3538-3546.

Hoogenboom, H.R., Griffiths, A.D., Johnson, K.S., Chiswell, D.J., Hudson, P. and Winter, G. (1991). Multi-subunit proteins on the surface of filamentous phage. Methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Research* **19**, 4133-4137.

Hoogenboom, H.R. (1997). Mix and match: Building manifold binding sites. *Nature Biotechnology* **15**, 125-126.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholz, D., Rogers, S.G. and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. *Science* **227**, 1229-1231.

Horton, R.M., Cai, Z., Ho, S.N. and Pease, L.R. (1990). Gene splicing by overlap extension: Tailor-made genes using the polymerase chain reaction. *Bio/Techniques* **8**, 528-535.

Horwitz, A.H., Chang, C.P., Better, M., Hellstrom, K.E. and Robinson, R.R. (1988). Secretion of functional antibody and Fab fragment from yeast cells. *Proceedings of the National Academy of Sciences USA* **85**, 8678-8682.

Hu, S.Z., Shively, L., Raubitschek, A., Sherman, M., Williams, L.E., Wong, J.Y.C., Shively,J.E. and Wu, A.M. (1996). Minibody: A novel engineered anti-carcinoembryonic antigen antibody fragment (single-chain Fv-C-H3) which exhibits rapid, high-level targeting of xenografts. *Cancer Research* **56**, 3055-3061.

Huang, L., Franklin, A.E. and Hoffman, N.E. (1993). Primary structure and characterization of an *Arabidopsis thaliana* calnexin-like protein. *Journal of Biological Chemistry* **268**, 6560-6566.

Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., Crea, R. and Opermann, H. (1988). Protein engineering of antibody binding sites: Recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli. Proceedings of the National Academy of Sciences USA* **85**, 5879-5883.

Hwang, C., Sinskey, A.J. and Lodish, H.F. (1992). Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* **257**, 1496-1502.

Iturriaga, G., Jefferson, R.A. and Bevan, M.W. (1989). Endoplasmic reticulum targeting and glycosylation of hybrid proteins in transgenic tobacco. *Plant Cell* **1**, 381-390.

Jämsä, E., Simonen, M. and Makarow, M. (1994). Selective retention of secretory proteins in the yeast endoplasmic reticulum by treatment of cells with a reducing agent. *Yeast* **10**, 355-370.

Jiang, W.R., Venugopal, K. and Gould, E.A. (1995). Intracellular interference of tick-borne flavivirus infection by using a single-chain antibody fragment delivered by recombinant Sindbis virus. *Journal of Virology* **69**, 1044-1049.

Jones, J.D.G., Gilbert, D.E., Grady, K.L. and Jorgensen, R.A. (1987). T-DNA structure and gene expression in petunia plants transformed by *Agrobacterium tumefaciens* C58 derivatives. *Molecular and General Genetics* **207**, 478-485.

Jones, P.T., Dear, P.H., Foote, J., Neuberger, M.S. and Winter, G. (1986). Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* **321**, 522-525.

Jost, C.R., Kurucz, I., Jacobus, C.M., Titus, J.A., George, A.J.T. and Segal, D.M. (1994). Mammalian expression and secretion of functional single-chain Fv molecules. *Journal of Biological Chemistry* **269**, 26267-26273.

Julius, D., Brake, A., Blair, L., Kunisawa, R., and Thorner-J (1984). Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-alpha-factor. *Cell* **37**, 1075-1089.

Kabat, E.A., Wu, T.T., Reid-Miller, M., Perry, H.M. and Gottesman, K.S. (1987). Sequences of proteins of immunological interest. US Department of Health and Human Services, Washington DC.

Kabat, E.A., WU, T.T., Perry, H.M., Gottesman, K.S. and Foeller, C. (1991). Sequences of Proteins of Immunological Interest, 5th ed.. US Dept. of Health and Human Services, Washington.

Kay, R., Chan, A., Daly, M. and McPherson, J. (1987). Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science* **236**, 1299-1302.

Kellog . D.E., Rybalkin, I., Chen, S., Mukhamedova, N., Vlasik, T., Siebert, P.D., Chenchik, A. (1994). Taqstart antibody: "Hot start" PCR facilitated by a neutralizing monoclonal antibody directed against taq DNA polymerase. *Biotechniques* **16**, 1134-1137.

Köhler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting of predefined specificity. *Nature* **256**, 52-53.

Koncz, C. and Schell, J. (1986). The promoter of T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Molecular and General Genetics* **204**, 383-396.

Kornfeld, R. and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. *Annual Review of Biochemistry* **54**, 631-664.

Kuipers, A.G.J., Soppe, W.J.J., Jacobsen, E. and Visser, R.G.F. (1995). Factors affecting the inhibition by antisense RNA of granule-bound starch synthase gene expression in potato. *Molecular and General Genetics* **246**, 745-755.

Laemmli, U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lahiri, D.K. (1994). Effect of ionophores on the processing of the beta-amyloid precursor protein in different cell lines. *Cellular and Molecular Neurobiology* **14**, 297-313.

Langridge, W.H.R., Fitzgerald, K.J., Koncz, C., Schell, J. and Szalay, A.A. (1989). Dual promoter of *Agrobacterium tumefaciens* is regulated by plant growth hormones. *Proceedings of the National Academy of Sciences, USA* **86**, 3219-3223.

Lazo, G.R., Stein, P.A. and Ludwig, R.A. (1991). A DNA transformation-competent arabidopsis genomic library in *Agrobacterium. Bio/technology* **9**, 963-967.

Leung, J., Fukuda, H., Wing, D., Schell, J. and Masterson, R. (1991). Functional analysis of *cis*-elements, auxin response and early developmental profiles of the mannopine synthase bidirectional promoter. *Molecular and General Genetics* **230**, 463-474.

Lewis, M.J. and Pelham, H.R. (1990). A human homologue of the yeast HDEL receptor. *Nature* **348**, 162-163.

Lodish, H., Baltimore, D., Berk, A, Zipurski, S.L., Matsudaira, P. and Darnell, J. (1995) Molecular Cell Biology. W.H. Freeman and Company, N.Y.

Logemann, J., Schell, J. and Willmitzer, L. (1987). Improved method for the isolation of RNA from plant tissues. *Analytical Biochemistry* **163**, 16-20.

Machamer, C.E. and Rose, J.K. (1988). Influence of new glycosylation sites on expression of the vesicular stomatitis virus G protein at the plasma membrane. *Journal of Biological Chemistry* **263**, 5948-5954.

Maciejewski, J.P., Weichold, F., Young, N.S., Cara, A., Zella, D., Reitz Jr, M.S. and Gallo, R.C. (1995). Intracellular expression of antibody fragments directed against HIV reverse transcriptase prevents HIV infection in vitro. *Nature Medicine.* **1**, 667-673.

Marasco, W.A., Haseltine, W.A. and Chen, S.Y. (1993). Design, intracellular expression, and activity of a human anti-human immunodefficiency virus type 1 gp120 single-chain antibody. *Proceedings of the National Academy of Sciences USA* **90**, 7889-7893.

Melnick, J., Dul, J.L. and Argon, Y. (1994). Sequential interaction of the chaperones BiP and GRP94 with immunoglobulin chains in the endoplasmic reticulum. *Nature* 370, 373-375.

Mhashilkar, A.M., Bagley, J., Chen, S.Y., Szilvay, A.M., Helland, D.G. and Marasco, W.A. (1995). Inhibition of HIV-1 Tat-mediated LTR transactivation and HIV-1 infection by anti-fat single chain intrabodies. *EMBO Journal* **14**, 1542-1551.

Muesch, A., Hartmann, E., Rohde, K., Rubartelli, A., Sitia, R. and Rapoport, T.A. (1990). A novel pathway for secretory proteins? *Trends in Biological Science* **15**, 86-88.

Munro, S. and Pelham, H. (1986). An Hsp-70 like protein in the ER. identity with the 78 kD glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell* **46**, 291-300.

Nap, J.P., Keizer, P. and Jansen, R. (1993). First generation transgenic plants and statistics. *Plant Molecular Biology Reports* **11**, 156-165.

Neave, H.R. and Worthington, P.L. (1988). Distribution-free Tests. Unwin Hyman Ltd, London.

Neu, H.C. and Heppel, L.A. (1965). The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. *Journal of Biological Chemistry* **240**, 3685-3692.

Neuberger, M.S., Williams, G.T. and Fox, R.O. (1984).Recombinant antibodies possessing novel effector functions. *Nature* **312**, 604-608.

Neuberger, M.S., Williams, G.T., Mitchell, E.B., Jouhal, S.S., Flanagan, J.G. and Rabbitts, T.H. (1985). A hapten-specific chimaeric IgE antibody with human physiological effector function. *Nature* **314**, 268-70.

Nieba, L., Honegger A., Krebber, C. and Plückthun, A. (1997). Disrupting the hydrophobic patches at the antibody variable/constant domain interface: improved *in vivo* folding and physical characterization of an engineered scFv fragment. *Protein Engineering* **10**, 435-44.

Odell, J.T., Knowlton, S., Lin, W., Mauvais, C.J. (1988). Properties of an isolated transcription stimulating sequence derived from the cauliflower mosaic virus 35S promoter. *Plant Molecular Biology* **10**, 263-272.

Olsder, J. and Hermsen, J.G.T. (1976) Genetics of self-incompatibility in dihaploids of *Solanum tuberosum* L. 1 Breeding behaviour of two self-compatible dihaploids. *Euphytica* **25**, 597-607.

Orlandi, R., Güssow, D.H., Jones, P.T. and Winter, G. (1989). Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. *Proceedings of the National Academy of Sciences USA* **86**, 3833-3837.

Owen, M., Gandecha, A., Cockburn, B. and Whitelam, G. (1992). Synthesis of a functional anti-phytochrome single-chain Fv protein in transgenic tobacco. *Bio/technology* **10**, 790-794.

Pantoliano, M.W., Bird, R.E., Johson, S., Asel, E.D., Dodd, S.W., Wood, J.F. and Hardman, K.D. (1991). Conformational stability, folding, and ligand-binding affinity of single-chain Fv immunoglobulin fragments expressed in Escherichia coli. *Biochemistry* **30**, 10117-10125.

Parks, D., Bolinger, R. and Mann, K. (1997). Redox state regulates binding of p53 to sequence-specific DNA, but not to non-specific or mismatched DNA. *Nucleic Acids Research* **25**, 12899-1295.

Peach, C. and Velten, J. (1991). Transgene expression variability (position effect) of CAT and GUS reporter genes driven by linked divergent T-DNA promoters. *Plant Molecolar Biology* **17**, 49-60.

Pelham, H.R.B. (1989). Heat shock and the sorting of luminal ER proteins. *EMBO Journal* **8**, 3171-3176.

Pfeiffer, N.E., Mehlhaff, P.M., Wylie, D.E. and Schuster, S.M. (1987). Topographical separation of the catalytic sites of asparagine synthetase explored with monoclonal antibodies. *Journal of Biological Chemistry* **262**, 11565-11570.

Piñeiro, M., García-Olmedo, F. and Diaz, I. (1994). Redox modulation of the expression of bacterial genes encoding cysteine-rich proteins in plant protoplasts. *Proceedings of the National Academy of Sciences USA* **91**, 3867-3871.

Phillips, J., Artsaenko, O., Fiedler, U., Horstmann, Mock, H.-P., M ntz, K.. and Conrad, U. (1997).Seed-specific immunomodulation of absisic acid activity induces a developmental switch. *EMBO Journal* **16**, 4489-4496.

Plückthun, A. (1991). Antibody engineering: Advances from the use of Eschrichia coli expression systems. *Bio/technology* **9**, 545-551.

Reddy, P., Sparvoli, A., Fagioli, C., Fassina, G. and Sitia, R. (1996). Formation of reversible disulfide bonds with the protein matrix of the endoplasmic reticulum correlates with the retention of unassembled Ig light chains. *EMBO Journal* **15**, 2077-2085.

Rees, A.R., Staunton, D., Webster, D.M., Searle, S.J., Henry, A.H. and Pedersen, J.T. (1994). Antibody design: Beyond the natural limits. *Trends in Biotechnology* **12**, 199-206.

Rennenberg, H., Filner, P. (1982). Stimulation of H2S emission from pumpkin leaves by inhibition of glutathione synthesis. *Plant Physiol.* **69**, 766-770.

Rodríguez-García, M.I., Fernández, M.C. and Alché J.D. (1995). Immunocytochemical localization of allergenic protein (*Ole e I*) in the endoplasmic reticulum of the developing pollen grain of olive (*Olea europaea* L.). *Planta* **196**, 558-563.

Rosso, M.-N., Schouten, A., Roosien, J., Borst-Vrenssen, T., Hussey, R.S., Gommers, F.J., Bakker, J., Schots, A. and Abad, P. (1996). Expression and functional characterization of a single chain FV antibody directed against secretions involved in plant nematode infection process. *Biochemical and Biophysical Research Communications* **220**, 255-263.

Rubartelli, A., Cozzolino, F., Talio, M. and Sitia, R. (1990). A novel secretory pathway for interleukin-1α, a protein lacking a signal sequence. *EMBO Journal* **9**, 1503-1510.

Rubartelli, A., Bajetto, A., Allavena, G., Wollman, E. and Sitia, R. (1992). Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *Journal of Biological Chemistry* **267**, 24161-24164.

Ruddon, R.W. and Bedows, E. (1997). Assisted protein folding. *Journal of Biological Chemistry* **272**, 3125-3128.

Ryabova, L.A., Desplancq, D., Spirin, A.S. and Plückthun, A. (1997). Functional antibody production using cell-free translation: Effects of protein disulfide isomerase and chaperones. *Nature Biotechnology* **15**, 79-84.

Salinas, J. (1992). Function of cutinolytic enzymes in the infection of Gerbera flowers by *Botrytis cinerea*. PhD thesis, University of Utrecht, The Netherlands.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: A laboratory manual, 2nd Edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sambamurti, K., Shioi, J., Anderson, J.P., Pappolla, M.A. and Robakis, N.K. (1992). Evidence for a intracellular cleave of the Alzheimer's amyloid precursor in PC12 cells. *Journal of Neuroscience Research* **33**, 319-329.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with the chain-terminating inhibitors. *Proceedings of the National Academy of Sciences USA* **74**, 5463-5467.

Schneider, W.P., Wensel, T.G., Stryer, L. and Oi, V.T. (1988). Genetically engineered immunoglobulins reveal structural features controlling segmental flexibility. *Proceedings of the National Academy of Sciences USA* **85**, 2509-2513.

Schots, A., de Boer, J., Schouten, A., Roosien, J., Zilverentant, J.F., Pomp, H., Bouwman-Smits, L., Overmars, H., Gommers, F.J., Visser, B., Stiekema, W.J. and Bakker, J. (1992). 'Plantibodies': a flexible approach to design resistance against pathogens. *Netherlands Journal of Plant Pathology* supplement 2, 183-191.

Schouten, A., Roosien, J., de Boer, J.M., Wilmink, A., Rosso, M.-N., Bosch, D., Stiekema, W.J., Gommers, F.J., Bakker, J. and Schots, A. (1997). Improving scFv antibody expression levels in the plant cytosol. *FEBS Letters* **415**, 235-241.

Schouten, A., Roosien, J., van Engelen, F.A., de Jong, G.A.M., Borst-Vrenssen, A.W.M., Zilverentant, J.F., Bosch, D., Stiekema, W.J., Gommers, F.J., Schots, A. and Bakker, J. (1996). The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco. *Plant Molecular Biology* **30**, 781-793.

Semenza, J., Hardwick, K.G., Dean, N. and Pelham, H.R. (1990). ERD2, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell* **61**, 1349-1357.

Shaheen, F., Duan, L.X., Zhu, M.H., Bagasra, O. and Pomerantz, R.J. (1996). Targeting human immunodeficiency virus type 1 reverse transcriptase by intracellular expression of single-chain variable fragments to inhibit early stages of the viral life cycle. *Journal of Virology* **70**, 3392-3400.

Shakin-Eshleman, S.H., Spitalnik, S.L. and Kasturi, L. (1996). The amino acid at the X position of an Asn-X-Ser sequon is an important determinant of N-linked core-glycosylation efficiency. *Journal of Biological Chemistry* **271**, 6363-6366.

Shin, S.U. and Morrison, S.L. (1989). Production and properties of chimeric antibody molecules. In: Langone, J.J. (Ed.), Methods in Enzymology, Vol. 178. Academic Press, San Diego, Ca. P.459-527.

Shirasu, Y., Shimada, Y., Takemura, K., Izumi, T and Miyazaki, H. (1988). Production and characterization of monoclonal antibodies to human pancreatic elastase 2. *Hybridoma* **7**, 485-493.

Sijmons, P.C., Dekker, B.M.M., Schrammeijer, B., Verwoerd, T.C., van den Elzen, P.J.M. and Hoekema, A. (1990). Production of correctly processed human serum albumin in transgenic plants. *Bio/technology* **8**, 217-221.

Singh, P., Tang, B.L., Wong, S.H. and Hong, W. (1993). Transmembrane topology of the mammalian KDEL receptor. *Molecular Cell Biology* **13**, 6435-6441.

Skerra, A. and Plückthun, A. (1988). Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli. Science* **240**, 1038-1041.

Smant, G., Stokkermans J.P.W.G., Yan, Y.T., deBoer, J.M., Baum, T.J., Wang, X.H., Hussey, R.S., Gommers, F.J., Henrissat, B., Davis, E.L., Helder, J., Schots, A. and Bakker, J. (1998) Endogenous cellulases in animals: Isolation of β-1,4-endoglucanase genes from two species of plant-parasitic cyst nematodes. *Proceedings of the National Academy of Sciences USA* **95**, 4906-4911.

Smith, R.G. and Baumgarten, B. (1987). Monoclonal antibodies that recognize a broad range of mammalian terminal deoxynucleotidyl transferases. *Hybridoma* **6**, 29-45.

Smith, R.I.F. and Morrison, S.L. (1994). Recombinant polymeric IgG: An approach to engineering more potent antibodies. *Bio/technology* **12**, 683-688.

Smith, M.D. (1996). Antibody production in plants. *Biotechnology Advances* **14**, 267-281.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150**, 76-85.

Staal, F.J.T., Roederer, M., Herzenberg, L.A. and Herzenberg, L.A. (1990). Intracellular thiols regulate activation of nuclear factor kappa-B and transcription of human immunodeficiency virus. *Proceedings of the National Academy of Sciences USA* **87**, 9943-9947.

Stieger, M., Neuhaus, G., Momma, T., Schell, J. and Kreuzaler, F. (1991). Self assembly of immunoglobulins in the cytoplasm of the alga *Acetabularia mediterranea*. *Plant Science* **73**, 181-190.

Sturm, A., Van Kuik, A., Vliegenthart, J.F.G. and Chrispeel, M.J. (1987). Structure, position, and biosynthesis of the high mannose and the complex oligosaccharide side chains of the bean storage protein phaseolin. *Journal of Biological Chemistry* **262**, 13392-13403.

Szederkényi, J., Komor, E. and Schobert, C. (1997). Cloning of the cDNA for glutaredoxin, an abundant sieve-tube exudate protein from *Ricinus communis* L. and characterisation of the glutathione-dependent thiol-reduction system in sieve tubes. *Planta* **202,** 349-356.

Tang, B.L., Wong, S.H., Low, S.H., Subramaniam, V.N. and Hong, W.J. (1994). Cytosolic factors block antibody binding to the C-terminal tail of the KDEL receptor. *European Journal of Cell Biology* **65**, 298-304.

Tatu, U., Braakman, I. and Helenius, A. (1993). Membrane glycoprotein folding, oligomerization and intracellular transport: Effects of dithiothreitol in living cells. *EMBO Journal* **12,** 2151-2157.

Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A. and Galeffi P. (1993). *Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from* virus attack. *Nature* **366**, 469-472.

Teeri, T.H., Lehväslaiho, H., Franck, M., Uotila, J., Heino, P., Palva, E.T., Van Montagu, M. and Herrera-Estrella, L. (1989). Gene fusions to *lacZ* reveal new expression patterns of chimeric genes in transgenic plants. *EMBO Journal* **8**, 343-350.

Thornton, J.M. (1981). Disulphide bridges in globular proteins. *Journal of Molecular Biology* **151,** 261-287.

Tretter V., Altmann, F., Maerz, (1991). Peptide-N-4-(N-acetyl-beta-glucosaminyl) asparagine amidase F cannot release glycans with fucose attached α1-3 to the asparagine-linked N-acetylglucosamine residue. *European Journal of Biochemistry* **199**, 647-652.

Tsumoto, K., Nakaoki, Y., Ueda, Y., Ogasahara, K., Yutani, K., Watanabe, K., Kumabai, I. (1994). Effect of the order of antibody variable regions on the expression of the single-chain HyHEL10 Fv fragment in E. coli and the thermodynamic analysis of its antigen-binding properties. *Biochemical and Biophysical Research Communications* 201, 546-551.

Valetti, C. and Sitia, R. (1994). The differentialeffects if dithiothreitol and 2-mercaptoethanol on the secretion of partially and completely assembled immunoglobulins suggest that thiol-mediated retention does not take place in or beyongd the Golgi. *Molecular Biology of the Cell* **5**, 1311-1324.

Vandenbosch, K.A. (1991) in: Electron Microscopy of Plant Cells. (eds. Hall, J.L. & Hawes, C.), pp. 181-218. London: Academic Press.

van Engelen, F.A., Schouten, A., Molthoff, J.W., Roosien, J., Salinas, J., Dirkse, W.G., Bakker, J., Gommers, F.J., Jongsma, M.A., Bosch, D. and Stiekema, W.J. (1994). Coordinate expression of antibody subunit genes yields high levels of functional antibodies in roots of transgenic tobacco. *Plant Molecular Biology* **26**, 1701-1710.

van Engelen, F.A., Molthoff, J.W., Conner, A.J., Nap, J.P., Pereira, A. and Stiekema, W.J. (1995). pBINPLUS: An improved plant transformation vector based on pBIN19. *Transgenic Research* **4**, 288-290.

Vaux, D., Tooze, J. and Fuller, S. (1989). Identification of an intercellular receptor for the KDEL retention signal. *Journal of Cell Biology* **109**, 99a.

von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Research* **14**, 4683-4690.

Voss, A., Niersbach, M., Hain, R., Hirsch, H.J., Liao, Y.C., Kreuzaler, F., and Fischer, R. (1995) Reduced virus infectivity in *N. tabacum* secreting a TMV-specific full-size antibody. *Molecular Breeding* **1**, 39-50.

Walther-Larsen, H., Brandt, J., Collinge, D.B. and Thordal-Christensen, H. (1993). A pathogen-induced gene of barley encodes a HSP90 homologue showing striking similarity to vertebrate forms resident in the endoplasmic reticulum. *Plant Molecular Biology* **21**, 1097-1108.

Wandelt, C.I., Khan, M.R.I., Craig, S., Schroeder, H.E., Spencer, D. and Higgins, T.J.V. (1992). Vicilin with carboxy-terminal KDEL is retained in the endoplasmic reticulum and accumulates to high levels in the leaves of transgenic plants. *Plant Journal* **2**, 181-192.

Werge, T.M., Baldari, C.T. and Telford, J.L. (1994). Intracellular single chain Fv antibody inhibits Ras activity in T-cell antigen receptor stimulated Jurkat cells. *FEBS Letters* **351**, 393-396.

Werge, T.M., Biocca, S. and Cattaneo, A. (1990). Intracellular immunization: Cloning and intracellular expression of a monoclonal antibody to the p21ras protein. *FEBS Letters* **274**, 193-198.

Wilnow, T.E., Moehring, J.M., Inocencio, N.M., Moehring, T.J. and Herz, J. (1996). The low-density-lipoprotein receptor-related protein (LRP) is processed by furin *in vivo* and *in vitro*. *Biochemical Journal* **313**, 71-76.

Whitelam, G.C. and Cockburn, W. (1996). Antibody expression in transgenic plants. *Trends in Plant Science* **1**, 268-272.

Whitlow, M., Bell, B.A., Feng, S.L., Filpula, D., Hardman, K.D., Hubert, S.L., Rollence, M.L., Wood, J.F., Schott, M.E., Milenic, D.E., Yokota, T. and Schlom, J. (1993). An improved linker for single-chain Fv with reduced aggregation and enhanced proteolytic stability. *Protein Engineering* **6**, 989-995.

Wilmink, A., Molthoff, J.W., Schouten, A., Roosien, J., Schots, A., Bakker, J., Stiekema, W.J and Bosch, D. (1998). Expression and localization of antibodies in roots of potato during infection by the potato cyst nematode. In preparation.

Wingate, V.P.M., Lawton, M.A., Lamb, C.J. (1988). Glutathione causes a massive and selective induction of plant defense genes. *Plant Physiology* **87**, 206-210.

Winter, G. and Milstein, C. (1991). Man-made antibodies. *Nature* **349**, 293-299.

Wood, C.R., Boss, M.A., Kenten, J.H., Calvert, J.E., Roberts, N.A. and Emtage, J.S. (1985). The synthesis and *in vivo* assembly of functional antibodies in yeast. *Nature* **314**, 446-449.

Wu, Y., Duan, L.X., Zhu, M.H., Hu, B.C., Kubota, S., Bagasra, O. and Pomerantz, R.J. (1996). Binding of intracellular anti-Rev single chain variable fragments to different epitopes of human immunodeficiency virus type 1 Rev: Variations in viral inhibition. *Journal of Virology* **70**, 3290-3297.

Yasui, H., Ito, W. and Kurosawa, Y. (1994). Effects of substitutions of amino acids on the thermal stability of the Fv fragments of antibodies. *FEBS Letters* **353**, 143- 146.

Ziegler, D.M. (1985). Role of reversible oxidation-reduction of enzyme thiol-disulfides in metabolic regulation. *Annual Review of Biochemistry* **54**, 305-329.

zu Putlitz, J., Kubasek, W.L., Duchêne, M., Marget, M., von Specht, B.-U. and Domdey, H. (1990). Antibody production in baculovirus-infected insect cells. *Bio/technology* **8**, 651-654.

Samenvatting.

Waardplantresistentie is een milieuvriendelijke methode voor de beheersing van ziekten en plagen, en is van oudsher een belangrijke eigenschap geweest waar veredelaars op selecteerden. Door de komst van de DNA technologie is het mogelijk geworden het arsenaal van resistente rassen drastisch uit te breiden en het gebruik van pesticiden verder terug te dringen. Deze technologie onderscheidt zich van de klassieke resistentieverdeling, doordat de soortsbarrière doorbroken wordt. Nieuwe eigenschappen kunnen worden geïntroduceerd die van origine niet in een plantensoort voorkomen. Introductie van natuurlijke resistentiegenen is te prefereren. Echter, hiervan is slechts een beperkt spectrum beschikbaar. Een alternatief is het ontwerpen van nieuwe genen.
Een nieuwe ontwikkeling in de moleculaire resistentieveredeling is het transformeren van planten met genen die coderen voor antilichamen. Antilichamen kunnen diverse biologische functies blokkeren en zijn hierdoor uitermate geschikt voor het creëren van nieuwe vormen van resistentie. De idee is dat de door de plant gesynthetiseerde antilichamen ('plantibodies') specifiek essentiele factoren (bijv. enzymen) van de parasiet of het pathogeen kunnen remmen. Bij deze benadering is het van cruciaal belang dat het antilichaam zich op dezelfde subcellulaire locatie bevindt als de te blokkeren pathogeniteitsfactor.
In dit proefschrift zijn de mogelijkheden onderzocht met betrekking tot de *in planta* expressie van antilichamen en antilichaamfragmenten en het versturen van deze eiwitten naar de juiste subcellulaire locaties.

Antilichamen, of immunoglobulinen, spelen een essentiële rol in het immuunsysteem van gewervelde dieren. Hun unieke functie is binding aan een schier eindeloze variatie van indringers (antigenen) met als doel om deze te neutraliseren en uiteindelijk te elimineren. Een antilichaam is een eiwit dat een Y-vormige structuur heeft en bestaat uit vier polypeptide ketens, twee identieke lichte ketens en twee identieke zware ketens (Fig. 1). Zowel de lichte als de zware keten hebben een duidelijk herkenbare domeinstructuur. Een lichte keten bestaat uit twee domeinen, een variabel en een constant domein. Een zware keten bestaat uit vier domeinen, een variabel domein en drie constante domeinen. De lichte keten is geassocieerd met het variabele domein en het eerste constante domein van de zware keten via een zwavelbrug en niet-covalente interacties. De twee zware ketens zijn, eveneens via zwavelbruggen en niet-covalente interacties, onderling geassocieerd via hun tweede en derde constante domeinen. De twee variabele domeinen van de lichte en zware keten vormen samen de bindingsplaats voor een antigeen. Ieder antilichaam is dus in staat twee antigenen te binden. Binding van een antilichaam heeft vaak tot gevolg dat het functioneren van het antigeen (bijv. een enzym) gedeeltelijk of volledig wordt geremd.

In hoofdstuk 2 is een effectieve methode beschreven om de twee genen, coderend voor de lichte en zware keten van een model antilichaam, te introduceren in planten en evenredig tot expressie te brengen. Beide genen bevinden zich op één transformatievector en worden op deze wijze simultaan in het plantengenoom geïntroduceerd. Op deze wijze zijn tijdrovende kruisingsprocedures of de moeizame methode van co-transformatie te vermijden. is een Een bijkomend voordeel dat uitsplitsing van beide genen bij kruising tijdens verdere veredeling uitgesloten is omdat beide genen op dezelfde chromosomale positie zijn geïncorporeerd. Om mogelijke 'silencing' effecten te vermijden zijn de twee genen onder controle gezet van

verschillende promoters. De resultaten tonen aan dat transgene planten de genen coderend voor lichte en zware ketens evenredig en op een relatief hoog niveau tot expressie brengen. Zoals verwacht worden de antilichamen gesecreteerd omdat een signaal voor translocatie naar het endoplasmatisch reticulum (ER) is toegevoegd.

Volledige antilichamen zijn dus uitermate geschikt om door plantencellen te worden uitgescheiden in de apoplast. Intracellulaire expressie van antilichamen is aanzienlijk gecompliceerder. In de reducerende omgeving van het cytosol is intramoleculaire zwavelbrugvorming, noodzakelijk voor de assemblage lichte en zware ketens, niet mogelijk. 'Single-chain Fv' (scFv) antilichaamfragmenten (Fig. 1) kunnen voor dit probleem uitkomst bieden omdat deze fragmenten geen intramoleculaire zwavelbruggen nodig hebben voor assemblage.
Antilichamen hebben een domeinstructuur die het mogelijk maakt om antilichamen op verschillende manieren te trimmen zonder dat dit de binding met het antigeen beïnvloedt (Fig. 1). Genen coderend voor antilichaamfragmenten zijn niet alleen in dierlijke cellen tot expressie gebracht, alsmede in gist en bacteriën. Eén van de meest interessante antilichaamfragmenten is een zogenaamd 'single-chain Fv' (scFv). Dit is een Fv fragment waarbij de variabele domeinen van de lichte en zware keten covalent verbonden zijn via een peptide 'linker' (Fig. 1). Hierdoor is een scFv veel stabieler dan een Fv fragment. Een bijkomend voordeel is dat een scFv gecodeerd wordt door één gen.
In hoofdstuk 3 is gekeken naar hoe efficient een scFv antilichaam zowel intra- als extracellulair tot expressie is te brengen in planten. Planten zijn getransformeerd met genen coderend voor een scFv antilichaam voorzien van signalen voor secretie, retentie in het

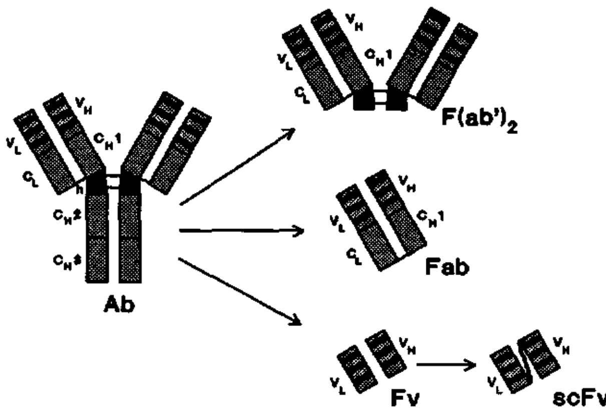

Fig. 1. Schematische weergave van een compleet antilichaam (Ab) en afgeleide antilichaamfragmenten ($F(ab')_2$, Fab, Fv en scFv). De variabele (V_L an V_H) en constante (C_L, C_H1, C_H3 en C_H3) domeinen binnen de lichte en zware keten, alsmede het hinge gebied (h), zijn weergegeven. Zwavelbruggen en niet covalente interacties houden de afzonderlijke peptiden ketens bij elkaar. $F(ab')_2$ en Fab fragmenten kunnen zowel via enzymatisch klieving als genetische manipulatie gemaakt worden. 'Single-chain Fv' (scFv) fragmenten zijn afgeleid van Fv fragmenten. Voor extra stabiliteit zijn de V_L an V_H domeinen covalent verbonden via een peptide linker. Zowel Fv als scFv fragmenten kunnen alleen verkregen worden via genetische manipulatie.

endoplasmatisch reticulum (ER) en expressie in het cytosol. Teleurstellend is dat secretie van de scFv leidt tot een relatief laag eiwitniveau. Het accumulatieniveau is relatief hoog indien de scFv intracellulair wordt vastgehouden in het endoplamatisch reticulum middels het ER retentie signaal, wat bestaat uit de vier aminozuren Lys-Asp-Glu-Leu ('KDEL'). Zeer opmerkelijk is dat de scFv, bestemd voor het cytosol, alleen detecteerbaar is wanneer een KDEL sequentie is toegevoegd. Dit is niet te verklaren door een verhoogd transcriptieniveau.

In hoofdstuk 4 is de intracellulaire expressie van scFv antilichamen nader bestudeerd. De reden van het vehoogde eiwitexpressieniveau van het cytosolische scFv fragment met extra KDEL sequentie kan veschillende oorzaken hebben. Enerzijds is dit scFv fragment, middels de KDEL sequentie, minder gevoelig voor proteases. Anderzijds kan de KDEL sequentie op zichzelf als signaal voor een onjuiste translocatie naar het ER hebben gefunctioneerd. Localisatiestudies met behulp van immunoelectronenmicroscopie en biochemische markers sluiten deze laatste mogelijkheid echter uit. De cytosolische scFv met KDEL sequentie is inderdaad in het beoogde subcellulaire compartiment aanwezig. Klaarblijkelijk heeft de KDEL extensie een positief effect op de stabiliteit van het scFv fragment in het cytosol. Dit is verder bevestigd door aan te tonen dat deze extensie ook positief werkt op de cytosolische expressie van enkele andere scFv fragmenten.

In hoofstuk 5 is bij zowel de cytosolische als de ER gelocaliseerde scFvs de aanwezigheid van intramoleculaire zwavelbruggen onderzocht. Deze zwavelbruggen zijn belangrijk voor de functionaliteit van het molecuul. Zoals verwacht zijn in het ER de zwavelbruggen inderdaad aanwezig. Opmerkelijk is dat ook in het cytosol zwavelbrugvorming plaatsvindt in stabiele transformanten. Bovendien zijn scFvs in beide compartimenten gedeeltelijk gedimeriseerd middels intermoleculaire zwavelbrugvorming tussen de cysteïneresiduen in de linker.
Zwavelbrugvorming in het cytosol is opvallend. Tot nu toe werd aangenomen dat, in tegenstelling tot de '*secretory pathway*', dit subcellulaire compartiment te reducerend is voor de vorming van zwavelbruggen.

In hoofstuk 6 is het model scFv fragment op verschillende manieren gemodificeerd met als doel de secretie te verbeteren, zonder de functionaliteit te beïnvloeden. In transiënte assays brengt het modificeren van proteolytisch gevoelige plaatsen in de linker geen verbetering in stabiliteit en secretie. Het gebruik van de $(Gly_4Ser)_3$-linker, in combinatie met de V_L-V_H architectuur, resulteert wel in een verhoogde intracellulaire accumulatie, echter niet in een verbetering van de secretie. Waarschijnlijk wordt het transport ergens in de 'secretory pathway' tegen gehouden, mogelijk door een kwaliteitscontrole mechanisme m.b.t. eiwitvouwing. Stabiele introductie van dit scFv-construct in transgene tabaksplanten resulteert echter niet in een hogere expressie en dus ook niet in een tegenomen secretie.
Een mogelijke verklaring voor deze resultaten is dat de variabele domeinen de stabiliteit van het scFv fragment bepalen. Indien de vouwing van deze domeinen onvoldoende stabiel is kunnen proteasen bepaalde gevoelige plaatsen nog steeds bereiken. Het C-terminaal toevoegen van constante domeinen kan mogelijk gevoelige plaatsen afschermen. Hiervoor zijn het CH_4 domein van een IgM antilichaam en de 'long hinge' (LH) en 'short hinge' (SH) van verschillende kamelenantilichamen gebruikt (Fig. 2). Beide hinges zijn staafvormig en vervangen het eerste constante domein van een normaal antilichaam. Bovendien ontbreken bij deze type kamelenantilichamen de lichte ketens en hebben beide hinges geen interactie met een ander domein nodig voor stabiliteit. Gekoppeld aan een scFv bevinden deze

domeinen zich dan ook in een vergelijkbare situatie als in kamelenantilichamen. Het CH_4 domein en beide hinges zouden bovendien kunnen zorgen voor efficiente multimerisatie (Fig. 2) wat een bijkomende verhoging van de schijnbare affiniteit (aviditeit) tot gevolg heeft. In stabiele transformanten blijkt alleen het toevoegen van het CH_4 domein de scFv expressie te verhogen. Dit resulteert weer in een verhoogde intracellulaire accumulatie zonder een verbetering van de secretie. Opmerkelijk is dat het toevoegen van een combinatie van de LH en het CH_4 domein wel leidt tot een verbeterde secretie. Deze scFv wordt zowel in monomere als dimere vorm gesecreteerd. De resterende intracellulaire accumulatie is in dit geval vermoedelijk veroorzaakt door een inefficiënte glycosylering van het CH_4 domein, hetgeen resulteert in een langer verblijf in het ER. Dit scFv construct heeft de potentie om naast dimeren ook complexere multimeren te vormen. In stabiele transformanten zijn deze echter niet aangetroffen.

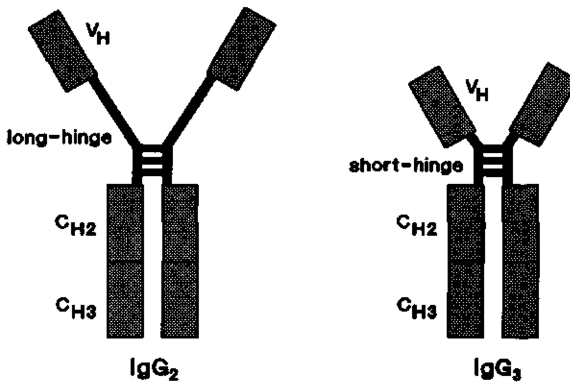

Fig. 2. Bij IgG_2 en IgG_3 antilichamen van kameel zijn de eerste constante domeinen (C_H1) vervangen door respectievelijk de 'long hinge' en 'short hinge'. Daarnaast zijn de lichte ketens in hun geheel afwezig.

Verdere optimalisatie zou uiteindelijk kunnen leiden tot een nog efficiëntere secretie van, scFv fragmenten, al of niet in multimere vorm. Hierbij kan ook nog gedacht worden aan alternatieve constructen zoals zogenaamde bivalente scFv fragmenten. Hierbij zijn twee scFv fragmenten covalent verbonden door een additionele linker. De eerste nog niet gepubliceerde resultaten tonen aan dat planten ook deze constructen tot expressie kunnen brengen.

Dit proefschrift toont aan dat antilichaamgenen redelijk eenvoudig gemodificeerd kunnen worden om een zo optimaal mogelijke intra- en extracellulaire expressie in planten te bereiken. Het potentieel van de plantibody-benadering is reeds aangetoond door de eerste voorbeelden van resistentie tegen tabaksmozaïekvirus (TMV) 'artichoke mottled crinkle virus' (ACMV) en 'beet necrotic yellow vein virus' (BNYVV). Van groot belang is het vinden van de geschikte 'target'-eiwitten. Indien deze voorhanden zijn dan lijkt het ontwerpen van resistente rassen met behulp van de plantibody-technologie niet alleen tegen virusen maar ook tegen nematoden, schimmels en insecten heel goed mogelijk.

Dankwoord/ Acknowledgements

Onderzoek doe je niet in je eentje en veel mensen hebben dan ook bijgedragen aan de totstandkoming van dit proefschrift.
Mijn dank gaat natuurlijk uit naar Jaap Bakker, Fred Gommers en Arjen Schots, een drie-eenheid die voor mij, en wellicht menig ander, soms moeilijk te scheiden is. Zakelijk gezien, jullie hebben gezorgd voor de twee projecten waaraan ik heb mogen werken. Belangrijker is dat jullie mij en Jan Roosien voor een groot deel de ruimte en het vertrouwen hebben gegeven het onderzoek binnen de kaders van de projecten naar eigen inzicht in te richten en uit te voeren. Ik ben zeer bewust van het feit dat ik mij op dat gebied in een luxe positie heb bevonden. Daarbij zorgden jullie altijd voor veel ondersteuning vanuit de vakgroep Nematologie en het Laboratorium voor Monoklonale Antistoffen (LMA). Dan was er nog het sociale gebeuren wat het onderzoek stimuleerde: je badend in het zweet door Jaap en Arjen mee laten sleuren over de cols van Pyreneeën en Alpen, overlevend op koude pizza en bier. Fred, jij voelt nu op je sloffen aan dat deze beeldvorming lichtelijk overtrokken is.
Jan Roosien, beste JanR, beste J.R., jij was mijn steun en toeverlaat. Al sinds de eerste kennismaking tijdens mijn afstudeervak Virologie doet jouw creativiteit en schier eindeloze parate kennis met betrekking tot moleculair onderzoek mij telkens weer versteld staan. Gelukkig ben ik niet de enige die dat zo ervaart. Jouw nooit aflatende positieve instelling is voor mij van groot belang geweest.
Ineke de Jong en Tanja Borst-Vrensen, jullie vormden een prachtig duo om mee te werken. De flair waarmee jullie samen het moleculaire werk uitvoerden is opvallend. Waar mogelijk hielpen jullie elkaar, ondanks de verschillende taken die jullie hadden. Dit resulteerde in een grote hoeveelheid waardevolle genconstructen, DNA-sequenties, planttransformaties, transiënte assays en western blots.
Jan de Boer wil ik bedanken voor het elektronenmicroscopische werk. De preparaten waren dusdanig goed gemaakt en geördend dat ik, na jouw vertrek naar de VS, alsnog de belangrijke EM plaatjes heb kunnen schieten voor hoofdstuk 4.
Marie-Noëlle Rosso, the French language is beautiful but still not easy. Saskia would have to dictate me if I dared to write in French. Therefore I stick to Dutch: 'Dank je wel voor jouw bijdrage aan het onderzoek.'
Fred van Engelen, jij had soms een frisse kijk op het onderzoek en het schrijven.
Jacoline Zilverentant wil ik bedanken voor het moleculaire werk en voor het zuiveren van menig 'monoklonaal' tijdens de eerste jaren van het onderzoek.
Petra Bakker, bedankt dat je mij de planttransformatie bij hebt willen brengen. Ik hoop dat ik je niet teveel voor de voeten heb gelopen op het CPRO.
Rikus Pomp, vooral het laatste jaar heb jij mij veel werk uit handen genomen zodat ik weer totaal nieuwe dingen kon gaan doen.
Lisette Groeneveld, jij zorgde dat de financiële overzichten en bestellingen met betrekking tot het 'Plantibody'-project altijd prima in orde waren. Ook de gevolgen van die irritante impulsaankopen van ons wist jij altijd weer in goede banen te leiden. Bedankt daarvoor.
Ik eindig in de privé-sfeer. Saskia en Thomas, bedankt dat jullie mij de ruimte hebben gegeven om dit proefschrift af te ronden. Jullie verdienen allebei meer aandacht. Saskia, de afgelopen tijd zei ik zo'n beetje iedere dag dat het proefschrift bijna af was. Ik weet dat jij je dan elke keer weer afvroeg wanneer het dan nu écht eens af zou zijn. Welnu, het is af.

Curriculum Vitae

Alexander Schouten werd geboren op 24 Oktober 1963 te Huizen. Na het behalen van het O.V.W.O. diploma aan Het Nieuwe Lyceum te Hilversum, begon hij in 1983 aan de studie plantenziektenkunde aan de Landbouwuniversiteit te Wageningen. De studie werd afgerond met afstudeervakken bij de vakgroepen Biochemie, Nematologie en Virologie en een stage bij het Department of Plant Pathology, University of Georgia, Athens, Georgia, U.S.A.. In 1989 behaalde hij zijn ingenieursdiploma. In oktober van hetzelde jaar begon hij als toegevoegd onderzoeker bij de vakgroep Nematologie, LUW, op het project "Onderzoek naar de overerving van virulentie en avirulentie en karakterisering van virulentie-genen en -mechanisme", gefinancierd door de Nederlandse Aardappel Associatie. Aansluitend werkte hij van juli 1991 tot maart 1998 bij dezelfde vakgroep als projectmedewerker voor NWO op het BION/STW-project "Moleculaire resistentieveredeling tegen aardappelcysteaaltjes met behulp van monoklonale antilichamen".

Sinds mei 1998 is hij als Post-doc werkzaam bij de leerstoelgroep Fytopathologie op het EEG project "Oxidative attack by necrotic pathogens – New approaches for an innovative and non-biocidal control of plant diseases".

Schots, A., Hermsen, T., Schouten, S., Gommers, F.J., & Eghberts, E. (1989). Serological differentiation of the potato cyst nematodes *Globodera pallida* and *G. rostochiensis*: II.Preparation and characterization of species specific monoclonal antibodies. *Hybridoma* **8**, 401-413.

Zuidema, D., Schouten, A., Usmany, M., Maule, A.J., Belsham,G.J., Roosien, J., Klinge-Roode, E.C., van Lent, J.W.M., and Vlak, J.M. (1990). Expression of cauliflower mosaic virus gene I in insect cells using a novel polyhedrin-based baculovirus expression vector. *J Gen Virol* **71**, 2201-2209.

Vlak, J.M., Schouten, A., Usmany, M., Belsham, G.J., Klinge-Roode, E.C., Maule, A.J., van Lent, J.W.M., and Zuidema, D. (1990). Expression of cauliflower mosaic virus gene I using a baculovirus vector based upon the p10 gene and a novel selection method. *Virology* **179**, 312-320.

Schots, A., Schouten, A., Roosien, J., Zilverentant, J.F.,Pomp, H., Bouwman-Smits, L., de Boer, J., Overmars, H.,Gommers, F.J., Visser, B., Stiekema, W.J. & Bakker, J. (1992). Plantibodies: a flexible approach to design resistance against pathogens. *Neth J Plant Pathol*, **98**, *suppl. 2*, 183-191.

Bakker, J. Bouwman-Smits, L., de Boer, J.M., Overmars, H.A., Roosien, J., Schouten, A., Gommers F.J., Janssen, R., Schots, A., Pomp, H., Zilverentant J.F., van Vloten-Doting L., Visser, B., Jongsma, M., Stiekema, W.J.,Gommers, F.J. Molecular strategies to study the host-parasite interaction between potato and Globodera rostochiensis (1992). In: Nematology from molecule to ecosystem: Proceedings Second International Nematology Congress, 11-17 August 1990, Veldhoven, the Netherlands. eds.: Gommers, F.J. and Maas, P.W.Th., pp 21-31.

van Engelen, F.A., Schouten, A., Molthoff, J.W., Roosien, J., Salinas, J., Dirkse, W.G., Schots, A., Bakker, J., Gommers, F.J., Jongsma, M.A., Bosch, D., Stiekema, W.J. (1994). Coordinate *expression of antibody subunit genes yields high levels of functional antibodies in roots of* transgenic tobacco. *Plant Mol Biol* **26**,1701-1710.

A. Schouten, J. Roosien, F.A. van Engelen, G.A.M. de Jong, A.W.M. Borst-Vrenssen, J.W. Molthoff, W.J. Stiekema, A. Schots and J. Bakker. Antibody mediated resistance against potato cyst nematodes. 22nd International Nematology Symposium, 7-12 August 1994, Gent, België, p. 119.

Rosso, M.-N., Schouten, A., Roosien, J., Borst-Vrenssen, T., Hussey, R.S., Gommers, F.J., Bakker, J., Schots, A., Abad, P. (1996). Expression and functional characterization of a single chain Fv antibody directed against secretions involved in plant nematode infection process. *Biochem Biophys Res Commun* **220**, 255-263.

Schouten, A., Roosien, J., van Engelen, F.A., de jong, G.A.M., Borst-Vrenssen, A.W.M., Zilverentant, J.F., Bosch, D., Stiekema, W.J.; Gommers, F.J., Schots, A., Bakker, J. (1996). The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco. *Plant Mol Biol* **30**, 781-793.

J. Roosien, A. Schouten, J.M. de Boer, G.A.M. de Jong, A.W.M. Borst-Vrenssen, A. Schots and J. Bakker. Influence of modifications in the linker peptide on the accumulation of scFv antibodies in plant cell compartments. Perspectives on Protein Engineering: 'From folds to functions', 2-6 March 1996, Montpellier, Frankrijk, p26.

A. Schouten, J. Roosien, J.M. de Boer, F.A. van Engelen, A. Wilmink, G.A.M. de Jong, A.W.M. Borst-Vrenssen, H. Pomp, H. Overmars, J.F. Zilverentant, J.W. Molthoff, D. Bosch, F.J. Gommers, W.J. Stiekema, A. Schots and J. Bakker. (Pl)antibodies; a possible alternative in the absence of natural host-plant resistance genes. 13th Triennial Conference of the European Association for Potato Research, 14-19 July 1996, Veldhoven, p. 701.

Schouten, A., Roosien, J., de Boer, J.M., Wilmink, A., Rosso, M.-N., Bosch, D., Stiekema, W.J.,Gommers, F.J.,Bakker, J. and Schots, A.(1997). Improving scFv antibody expression levels in the plant cytosol. *FEBS Lett* **415**, 235-241.

Alexander Schouten, Jan Roosien, Jan M. de Boer, Dirk Bosch, Arjen Schots and Jaap Bakker. (Pl)antibody mediated resistance against plant pathogens. 5th International Conference of Plant Molecular Biology, 21-27 September 1997, Singapore, abstract 316.

Alexander Schouten, Jan Roosien, Jan M. de Boer, Arjen Schots and Jaap Bakker. Functional expression of a single-chain Fv antibody in the plant cytosol: Formation of disulfide bridges in a reducing ectopic environment. 1997 Current Topics in Gene Expression Systems, 2 t/m 5 November 1997, San Diego, U.S.A., p. 20.

Stiekema, W.J., Bosch D., Wilmink A., De Boer J.M., Schouten A., Roosien J., Goverse A., Smant G., Stokkermans J.P.W.G., Gommers F.J., Schots A., Bakker J. (1997). Towards plantibody-mediated resistance against nematodes. In: Cellular and molecular aspects of plant-nematode interactions, Fenoll, C., F.M.W. Grundler, and S.A. Ohl (eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, pag. 262-272.

Schouten, A., Roosien, J., Bakker, J. and Schots, A. (1998). Disulfide bridge formation within and between single-chain Fv antibodies in the reducing ectopic environment of the plant cytosol. In preparation.

Roosien, J., Schouten, A.,de Jong, G.A.M., Borst-Vrenssen, A.W.M., Schots, A., Bakker, J. (1998). ScFv stability in the secretory pathway of plant cells: Influence of linker peptides, the scFv architecture, C_H1 residues and the addition of camel hinges and IgM C_H4 domains. In preparation.

Wilmink, A., Molthoff, J.W., Schouten, A., Roosien, J., Schots, A., Bakker, J., Stiekema, W.J and Bosch, D. (1998). Expression and localization of antibodies in roots of potato during infection by the potato cyst nematode. In preparation.