Plant Molecular Biology **43:** 419–428, 2000.

Mini-review

The plantibody approach: expression of antibody genes in plants to modulate plant metabolism or to obtain pathogen resistance

Geert De Jaeger, Chris De Wilde, Dominique Eeckhout, Esbjörn Fiers and Ann Depicker*
*Vakgroep Moleculaire Genetica, Departement Plantengenetica, Vlaams Interuniversitair Instituut voor Biotechnologie (VIB), Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium (*author for correspondence; e-mail: anna.depicker@rug.ac.be)*

Received 18 April 2000; accepted in revised form 18 May 2000

Key words: antibody production, gene inactivation, immunomodulation, phage display, plantibody, reducing environment, single-chain variable fragment

Abstract

Immunomodulation is a molecular technique that allows the interference with cellular metabolism or pathogen infectivity by the ectopic expression of genes encoding antibodies or antibody fragments. In recent years, several reports have proven the value of this tool in plant research for modulation of phytohormone activity and for blocking plant-pathogen infection. Efficient application of the plantibody approach requires different levels of investigation. First of all, methods have to be available to clone efficiently the genes coding for antibodies or antibody fragments that bind the target antigen. Secondly, conditions to obtain high accumulation of antigen-binding antibodies and antibody fragments in plants are being investigated and optimized. Thirdly, different strategies are being evaluated to interfere with the function of the target molecule, thus enabling immunomodulation of metabolism or pathogen infectivity. In the near future, optimized antibody gene isolation and expression, especially in reducing subcellular environments, such as the cytosol and nucleus, should turn immunomodulation into a powerful and attractive tool for gene inactivation, complementary to the classical antisense and co-suppression approaches.

Introduction

The fast-moving field of recombinant antibody engineering and expression continuously opens up new opportunities, not only for the medical sciences, but also for applied and fundamental agronomic research. Many reports describe how very efficiently transgenic plants produce complete antibodies or antibody fragments, such as Fab and single-chain variable (scFv) fragments (Figure 1) (for a review, see De Wilde *et al.*, 1999). Antibodies or antibody fragments produced in plants are often referred to as 'plantibodies' and they can be exploited for *ex planta* applications in the field of molecular farming. After isolation and purification from the plant tissue, the antibodies or antibody fragments can be used in industrial processes, as diagnostic tools, for immunochromatography, or in medical therapy (Figure 1). In addition, there is also a growing interest for *in planta* applications. Here, we describe a strategy, called immunomodulation, in which antibodies or antibody fragments are produced to modulate the function of a corresponding antigen. As such, immunomodulation can be used to study the function of the antigen or even of the epitope in plants, to change agronomic traits, or to 'immunize' the plant against pathogen infection (Figure 1). We highlight recent accomplishments in the use of plantibodies for immunomodulation. We comment on the progress in the isolation of recombinant antibody sequences and their expression in plants, and we propose future benchmarks that will further stimulate the general application of this promising engineering technology in plant molecular biology and agronomic research.

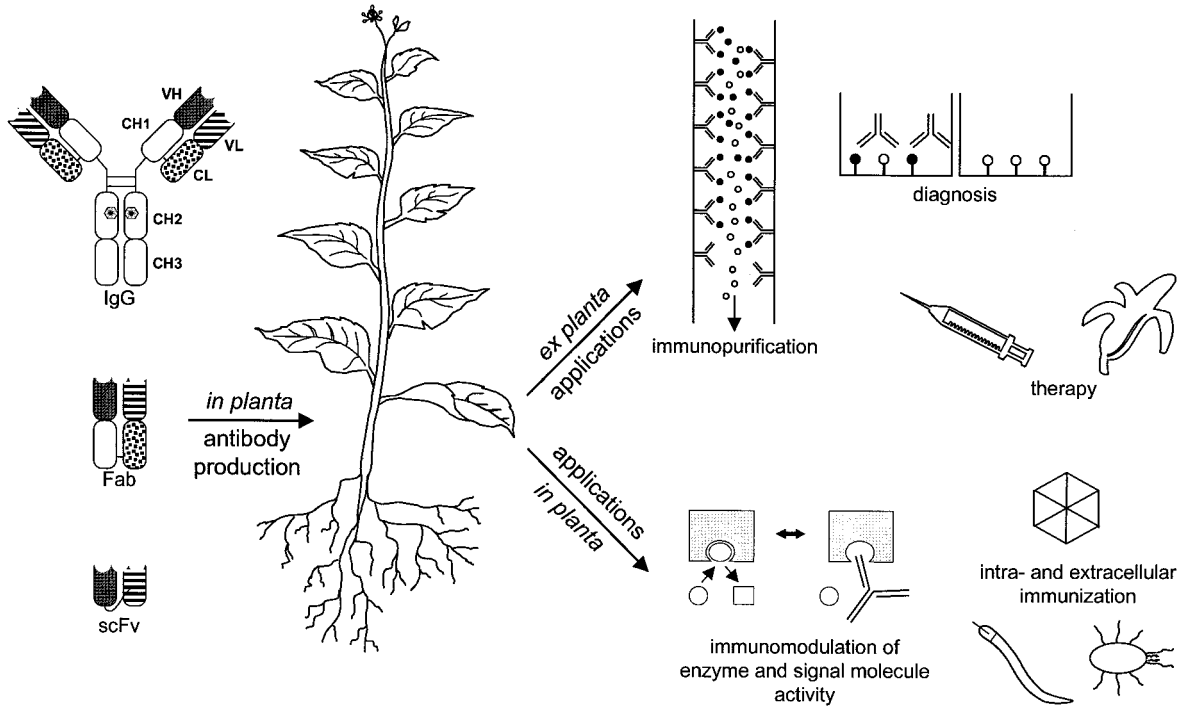

Figure 1. In planta and *ex planta* applications of antibodies and antibody fragments produced in plants. Plants can be engineered to synthesize antibodies in order to obtain pathogen resistance (intra- and extracellular immunization) or to manipulate the plant's metabolism, such as the immunomodulation of enzyme or signal molecule activity. Isolation and purification, if necessary, allows the use of plant-made antibodies for diagnosis, affinity-based purification, and therapy.

The concept of immunomodulation

In theory, antigens present in any subcellular compartment can be subjects of immunomodulation. So far, antibodies or antibody fragments in plants have been targeted to the cytosol, the endoplasmic reticulum (ER), and the apoplast (see below), but production of antibody fragments in the nucleus, mitochondria, or chloroplasts should also be possible. Antigen-antibody interaction *in vivo* could result in modulation of the antigen's activity in several ways (Figure 2). Binding of the antibody on the substrate or ligand-binding site of an enzyme or receptor blocks interactions between enzyme and substrate or between receptor and ligand directly by competitive inhibition (Figure 2(1)). Moreover, binding of the antibody on other sites of the enzyme or receptor can cause allosteric inhibition by inducing structural changes in the active site or ligand-binding site (Figure 2(2)). Another possibility is binding of the substrate or ligand itself (Figure 2(3)). Furthermore, antibody binding can interfere with the protein folding (Figure 2(4)), either directly or by hindering the interaction with chaperones. Additionally, the formation of quaternary protein structures, such as multienzyme complexes, can be prohibited (Figure 2(5)). Finally, the antigen can be mistargeted as a result of antibody binding (Figure 2(6)), such as retention in the ER of ER-translocated secretory proteins or metabolites.

Immunomodulation of endogenous targets in plants

The strategy of antigen mistargeting was applied to immunomodulate phytohormone activity. Tobacco plants accumulat scFv fragments in the ER that bind to abscisic acid (ABA) were shown to wilt under ambient conditions (Artsaenko *et al.*, 1995). These plants exhibited an increased transpiration rate, indicating that they were unable to close their stomata. In contrast with several wilty, ABA-deficient mutants, 2 to 10 fold higher ABA levels were found in scFv-accumulating than in wild-type plants. Most probably, this phenomenon can be explained by the fact that the concentrated scFv antibodies in the ER provide an ABA sink and prevent the transport of the hormone and its interaction with ABA receptors in the guard cells of the stomata

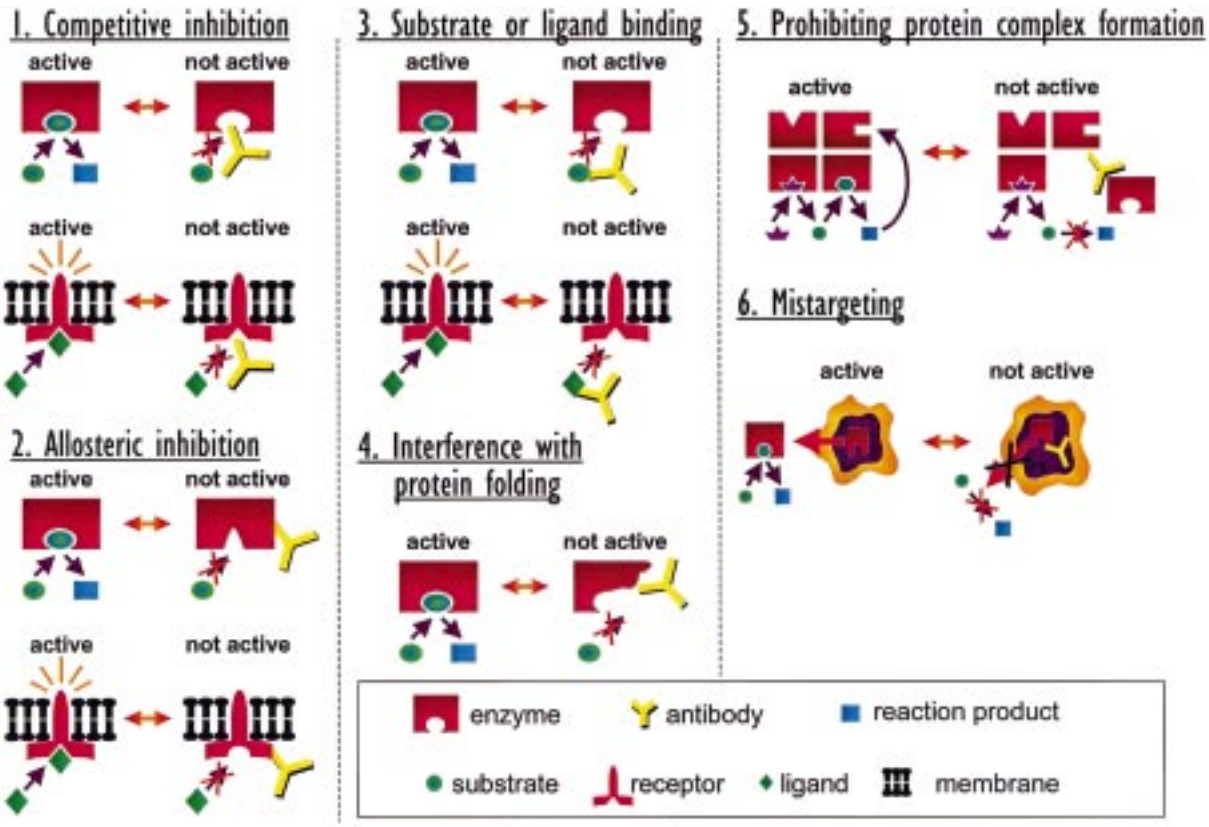

Figure 2. Potential mechanisms of antibody-mediated *in vivo* modulation of protein or signal molecule activity (for details, see text).

(Artsaenko *et al.*, 1995, 1999). Besides ubiquitous production of the anti-ABA scFv, seed-specific scFv production leads to modulation of ABA levels and activity in a tissue- or time-specific manner (Phillips *et al.*, 1997).

Recently, preliminary results have been reported on the expression of an anti-gibberellin A19/24 scFv fragment in tobacco (Shimada *et al.*, 1999). The gibberellins A19 and A24 are precursors of the bioactive gibberellins A1 and A4. Transgenic lines that accumulate high levels of the scFv in the ER showed a dwarf phenotype and lower gibberellin A1 levels than wild type. Because shoot elongation is a typical response regulated by gibberellins, the results suggest that scFv proteins reduced the concentration of bioactive gibberellins by trapping and inhibiting the metabolism of the bioactive gibberellin precursors A19 and A24. Together, these results show the value of the immunomodulation approach for the study and modulation of phytohormone activity. Artsaenko *et al.* (1999) point out an important advantage of immunomodulation versus the use of existing mutants or conventional antisense technology to study hormone action namely that antibody binding can inactivate an end product of a hormone biosynthetic pathway without affecting the function of any precursors. In contrast, in many biosynthetic mutants and during application of ABA inhibitors, ABA synthesis is blocked early in the pathway, thus making it difficult to attribute the observed physiological changes to the function of a particular endogenous regulator. In addition, the levels of inhibition of phytohormone activity differ also between individual transgenic plants due to variations in expression of the scFv gene in different transgenic plant lines. This mechanism may allow production of plants with different levels of biologically active phytohormones.

Owen *et al.* (1992) have demonstrated receptor activity modulation with an scFv directed against the plant regulatory receptor protein, phytochrome. Seeds from transgenic plants expressing the scFv gene displayed aberrant phytochrome-dependent germination. Other successful trials of enzyme activity modulation in plants have not been reported yet, despite

the success of this approach in mammalian cells (Marasco, 1995; Lener *et al.*, 2000 and references therein). In one study, the flower pigment synthesis pathway of *Petunia hybrida* was used as a model system to prove *in vivo* inhibition of dihydroflavonol-4-reductase (DFR) activity (De Jaeger *et al.*, 1999). DFR is one of the key enzymes in the flavonoid pathway and *dfr*$^{-}$ mutants were shown to be deficient in flower pigments. Five different DFR-specific scFv fragments were expressed in *Petunia hybrida* under control of the 35S cauliflower mosaic promoter, but no phenotype could be observed. Although this pathway in *Petunia* had proven its value as a model system to study gene inactivation by antisense or co-suppression, it was not a good choice for immunomodulation. The DFR enzyme resides in the epidermal cell layer of the petals and accumulates to very high levels, estimated to be at least 0.5% of the total soluble protein (TSP) amount. The scFv fragments, on the contrary, seemed to accumulate to low levels in the cytosol of the epidermal cells. Moreover, *Petunia* lines with only 5% of the wild-type *dfr* mRNA level still show a wild-type flower color phenotype (Huits *et al.*, 1994), preventing the visualization of the immunomodulation effect.

Intra- and extracellular immunization against plant-pathogen infection

A major goal of molecular breeding is the generation of transgenic plants that show enhanced resistance to pathogen infection. In this area, immunomodulation of factors playing a role in the infection process can prove very powerful. Depending on the intra- or extracellular accumulation of the antibody or antibody fragment, this approach is termed intra- or extracellular immunization. The validity of the concept was demonstrated by the production of transgenic tobacco plants that produced antibodies directed against a viral coat protein. These plants were resistant to artichoke mottled crinkle virus (Tavladoraki *et al.*, 1993) or tobacco mosaic virus (TMV) (Voss *et al.*, 1995). Antibody-mediated virus resistance is seen as an attractive alternative to the various forms of pathogen-derived resistance. In the latter case, unintended side effects, such as heteroencapsidation and recombination of viral genomes, cannot always be excluded. Most plant viruses are RNA viruses that replicate in the cytosol. As such, the highest resistance is expected by targeting the antibody to this cell compartment. Zimmermann *et al.* (1998) have evaluated and compared protection of tobacco plants against TMV infection by the expression of a TMV virion-binding scFv fragment in the cytosol and the apoplast. Even though much higher accumulation levels were obtained for the scFv targeted to the apoplast, the most dramatic reduction of necrotic local lesion numbers upon virus infection was observed in plants accumulating scFv fragments in the cytosol. Infectivity could be reduced by more than 90%. Moreover, several plant lines showed inhibition of systemic virus spread. In another study, a scFv fragment specific to the coat protein of beet necrotic yellow vein virus (BNYVV) was produced in transgenic *Nicotiana benthamiana* and its effect on the infection with BNYVV was analyzed (Fecker *et al.*, 1997). The scFv was produced in the cytosol or targeted to the apoplast by an N-terminal signal sequence. No scFv proteins could be detected in the cytosol. The apoplast-targeted scFv seemed, for unknown reasons, to be retained inside the cells, perhaps in the ER. Upon infection of these plants with the scFv residing in the ER, the average time needed for infection symptoms to appear was longer in the scFv-producing plants than in non-producing control plants. In addition, the scFv-producing plants were partially protected against the pathogenic effects exerted by the virus in the late stages of infection (Fecker *et al.*, 1997; Fecker and Koenig, 1999). It is surprising that the scFv proteins targeted to the secretory pathway were able to interfere with the cytosolic replication of the virus. Apparently, upon entering of the virus in disrupted cells, the virus particles may have come in contact with scFv proteins. Possibly higher degrees of resistance might have been obtained when the scFv would have been able to accumulate in the cytosol.

Besides using immunomodulation to obtain virus resistance, production of a scFv directed against the major membrane protein of the stolbur phytoplasma proved to be an elegant strategy to control mycoplasma (phytoplasma and spiroplasma) infection (Le Gall *et al.*, 1998). Pathogenic phytoplasmas and spiroplasmas are localized exclusively to the sieve tubes of the phloem tissue, into which they are inoculated by insect vectors. ScFv proteins were produced in tobacco and targeted to the apoplast with the bacterial pelB leader sequence. Transgenic tobacco shoots were grafted on a phytoplasma-infected tobacco root stock and grew free of symptoms, while untransformed tobacco shoots showed severe stolbur symptoms and eventually died. A similar strategy was tested against corn stunt spiroplasma (CSS), but no

distinct resistance could be observed when compared to control plants (Chen and Chen, 1998).

Plantibody-mediated resistance against complex eukaryotic, multicellular pathogens, such as nematodes, fungi, and insects, remains a major challenge. Alternative methods to control pests, such as the plantibody approach, would reduce pollution from synthetic pesticides and make agriculture more sustainable. Resistance against root-knot nematodes (Baum *et al.*, 1996; Rosso *et al.*, 1996), which is one of the world's most damaging agricultural pests, has been tested upon accumulation of the IgM antibody 6D4. IgM 6D4 binds a glycoprotein of unknown function in the stylet secretions of the root-knot nematode *Meloidogyne incognita*. After the nematode has migrated to the vascular tissue, it injects secretions into cells of the vascular cylinder. These secretions are considered to be the chemical signals that alter plant cell cycle and cell differentiation, ultimately leading to the development of giant cells on which the nematode feeds until the completion of its life cycle. Attempts to make tobacco resistant were carried out by producing heavy and light chains of the antibody targeting them to the secretory pathway, allowing assembly into IgG-like IgM subunits (Baum *et al.*, 1996). Although antibodies were found in the feeding galls, they had no effect on root-knot nematode parasitism. Most probably, the antibodies accumulated apoplastically whereas nematode stylet secretions were injected into the cytosol of the parasitized cells. Therefore, Rosso *et al.* (1996) investigated the accumulation of 6D4 antibody-derived scFv fragments in the cytosol of plant cells. Transient expression in tobacco protoplasts gave high cytosolic accumulation of the scFv, especially when fused to a C-terminal KDEL sequence. Since then, transgenic plants have been made, expressing the scFv fragment intracellularly. Preliminary results of the nematode reproduction assay after infection of the transgenic plants are very promising and indicate a significant reduction in the number of egg masses (Marie-Noëlle Rosso, personal communication).

The future isolation and characterization of key pathogenicity factors will be crucial to combat these complex pathogens. Worth mentioning here is the isolation of several β-1,4-endoglucanase genes from two species of plant-parasitic cyst nematodes, *Globodera rostochiensis* and *Heterodera glycines* (Smant *et al.*, 1998). These proteins are secreted by the subventral glands, which suggests that migration through the cell walls of plant roots involves enzymatic softening. Inhibition of these enzymes would probably hinder nematodes from reaching their destination. The same could hold true for certain enzymes secreted by fungi. Polyclonal antibodies against pectate lyase of the fungus *Colletotrichum gloeosporioides* could inhibit symptom development in the host plant (Wattad *et al.*, 1997). For insects, immunization of vertebrate hosts with antigens from the parasite has been shown to protect against hematophagous arthropods (East *et al.*, 1993; Wong and Opdebeeck, 1993). When such vertebrate insects ingest antibodies from their host, a large fraction remains functional in the insect's gut and a small fraction passes into the hemolymph (Lehane, 1996), both enabling the antibodies to bind their antigen and to interfere with the insect's metabolism. Similar observations have been made for herbivorous insects (Ben-Yakir and Shochat, 1996). These results suggest strongly that the plantibody approach would also work against insects.

Isolation of antibody and antibody fragment-encoding sequences

Despite the above-mentioned contributions, the plantibody approach remains less popular to inactivate genes than, for instance, antisense and co-suppression approaches. Antisense and co-suppression are technically quite simple and straightforward compared to the complexity and technological difficulty of isolating and identifying genes that encode antigen-specific antibodies. To make immunomodulation user-friendlier, further optimization of antibody gene isolation is needed. Antibody genes are usually isolated from monoclonal hybridoma cell lines. However, hybridoma technology is expensive and time-consuming and, in general, plant laboratories do not have the facilities to work with animal cell cultures. Phage display technology, on the other hand, allows the isolation of antigen-binding scFv-encoding sequences directly from established libraries or from the spleen of immunized mice. Phage display makes use of generally applied recombinant DNA techniques. The feasibility of this approach was shown by selecting five scFv fragments against the plant enzyme dihydroflavonol-4-reductase (De Jaeger *et al.*, 1997) and by the successful expression of these scFv fragments in transgenic *Petunia hybrida* (De Jaeger *et al.*, 1999). After immunization, a scFv phage display library is made from the immunized mice by PCR amplification of antibody-encoding sequences from B-lymphocytes.

The antibody fragments are produced in the periplasm of *Escherichia coli* and displayed on the pIII coat proteins of M13 bacteriophage. Through several rounds of panning, antigen-binding scFv clones are selected from the library. From the bacterial clones, antibody fragment-encoding sequences are available for further cloning in plant expression vectors. Phage display is cheaper and faster than making hybridoma cell lines and, most importantly, no animal cell cultures are involved. Using this approach, it is feasible to isolate antibody fragment-encoding sequences from immunized mice in about 4 months starting from immunization. However, even further improvements in antibody gene isolation are possible by exploiting recent developments in recombinant antibody engineering, which promise to bypass immunization. These advances have been achieved by constructing huge naive Fab and scFv phage display libraries (Griffiths *et al.*, 1994; Vaughan *et al.*, 1996; Sheets *et al.*, 1998; de Haard *et al.*, 1999; Sblattero and Bradbury, 2000) from which high-affinity antibody fragment-encoding sequences can be isolated in only a few weeks against any antigen. Although applications of such libraries have been limited largely to medical research, library construction strategies are being continuously optimized (Sheets *et al.*, 1998; de Haard *et al.*, 1999; Sblattero and Bradbury, 2000) and the construction of phage display libraries for use in plant biology seems feasible in the near future. Worth mentioning is also the *in vitro* production of antibody fragments by ribosome display (Hanes and Plückthun, 1997), which should allow even library construction in *Escherichia coli* to be bypassed. The unit selected in this system consists of the recombinant protein connected to the encoding RNA via the ribosome. Library construction is carried out by PCR, *in vitro* transcription, and *in vitro* translation. No transformation is necessary and very large libraries could become accessible in a single step. Moreover, ribosome display avoids the tedious alternation between *in vitro* and *in vivo* steps. Furthermore, after each selection round, additional genetic diversity can be generated by error-prone PCR, expanding the size of the sequence space that can be screened and thus increasing the chance to find a specific antibody with the desired specificity and high affinity.

Expression of antibody and antibody fragment genes in transgenic plants

Further improvements in antibody gene expression in plants are needed, especially for antibody accumulation in reducing environments, such as the cytosol. Although the maximum level of antibody accumulation depends on the plant species, on the intrinsic properties of the antibody itself, and also on the tissue (for a review, see De Wilde *et al.*, 1999), we may conclude from a long list of publications that full-size antibodies and Fab fragments are accumulated at high levels (more than 0.1% of TSP) upon secretion by adding an N-terminal ER signal sequence. On the contrary, for scFv fragments it seems that the highest levels (1–5% of TSP) are obtained when they are retained in the ER by adding an N-terminal signal sequence and a C-terminal KDEL retention signal. Secreted scFv fragments seem to accumulate at levels that are less predictable. Whereas some scFv fragments accumulate to levels of 0.5% of the TSP, many other scFv fragments accumulate to low or even undetectable levels. Recently, the low accumulation level of secreted scFv fragments has been improved by fusing a camel long-hinge region with a murine IgM CH4 domain (Schouten, 1998) or by making bispecific single-chain antibodies (Fischer *et al.*, 1999). In conclusion, for immunomodulation of target molecules present in the apoplast, IgGs or Fabs are the best option. For immunomodulation in the ER, scFv fragments are the best choice; however, IgGs and Fab fragments can also be used (K. Peeters, personal communication).

The cytosol is the compartment in which high antibody accumulation levels are the most difficult to obtain, although several examples of successful immunomodulation with antibody expression in the plant cytosol have been reported (Owen *et al.*, 1992; Tavladoraki *et al.*, 1993; Zimmermann *et al.*, 1998). It is generally assumed that the individual chains of a full-size antibody or a Fab fragment cannot be correctly assembled in the cytosol because of the lack of chaperones and/or of the reducing environment of this cell compartment, which prevents the formation of disulfide bridges. ScFv antibody fragments have a higher chance of being correctly folded in the cytosol because the two domains that make up an scFv are interconnected by a flexible peptide linker, avoiding the need for assembly of different chains and the formation of inter-chain disulfide bridges.

However, scFv fragments have two intra-chain disulfide bridges that are essential for correct folding, which is believed to be the reason why several scFv fragments could not be detected in the cytosol, even when the corresponding mRNA levels were normal. This observation indicates that the low protein accumulation levels are most probably the result of instability, inefficient synthesis, or folding of the proteins. Despite the reports of low scFv accumulation levels in the plant cytosol, high accumulation levels of some scFv fragments have been reported by others (Owen *et al.*, 1992; Tavladoraki *et al.*, 1993; De Jaeger *et al.*, 1999). Together, these results suggest that the scFv accumulation capacity in the cytosol is highly dependent on the intrinsic properties of the expressed scFv fragment. Therefore, strategies are being developed to engineer, isolate or identify stable scFv scaffolds. Interestingly, many scFv fragments seem to be stabilized at the protein level by the addition of the C-terminal peptide (DI)KDEL or KDEI, resulting in elevated accumulation levels in the cytosol (Schouten *et al.*, 1996, 1997; Rosso *et al.*, 1996). It has been hypothesized (Schouten *et al.*, 1997) that addition of the KDEL peptide sterically protects a region of the scFv protein that is susceptible to proteolysis, or that the peptide simply prevents C-terminal protein degradation, or that it stabilizes the protein by enhancing the number of amino acid interactions. Unfortunately, addition of a KDEL or KDEI tetrapeptide does not improve the cytosolic accumulation levels of every scFv protein (Schouten *et al.*, 1997). Nevertheless, De Jaeger *et al.* (1999) observed high cytosolic accumulation of several scFv fragments, isolated by phage display, upon expression in transgenic *Petunia hybrida* and found that all five antibody fragments analyzed accumulated to detectable levels in the cytosol. This striking finding is possibly due to the use of the phage display technology, whereas all the previously reported scFv fragments produced in plants were constructed starting from a hybridoma cell line, which means that they were selected based on the activity of the complete antibody. In contrast, phage display allows selection of functional scFv fragments and, concomitantly, more stable scFv scaffolds can be selected (De Jaeger et al., 1999). A similar result was found in the study of the cytosolic accumulation of 13 avian scFv fragments, obtained by phage display from immunized chicken. ScFv expression constructs were transformed into tobacco and upon regeneration 7 out of 13 scFv fragments could be detected by immunoblot, indicating high accumulation in the plant cytosol (Stefan Schillberg, personal communication). Another important consideration regarding accumulation of antibody in the cytosol is the functionality of the antibody fragment. Three scFv fragments were shown to be effective when produced in the cytosol, indicating that these scFv fragments were functional as antigen-binding proteins (Owen *et al.*, 1992; Tavladoraki *et al.*, 1993; Zimmermann *et al.*, 1998). However, not all scFv fragments that accumulate to high levels in the cytosol are necessarily functional. In some cases, the absence of proper disulfide bond formation could prevent some scFv fragments from adapting conformations that are necessary for antigen binding (De Jaeger *et al.*, 1999). Again, intrinsic properties of the scFv protein seem to determine these characteristics. Altogether, it is necessary to have a quick assay to analyze scFv fragments for their intracellular performance. Recently, two screening methods have been developed. The first makes use of transient expression of antibody genes by *Agrobacterium* vacuum infiltration in intact leaves (Kapila *et al.*, 1997; De Wilde *et al.*, 1998; De Jaeger *et al.*, 1999). Upon leaf extraction, the antibody fragment accumulation and antigen-binding activity can be easily assessed by protein gel blot, ELISA, or other molecular techniques. This method is a fast way to check antibody fragment performance in plants before starting transgenic experiments. The second is an *in vivo* assay that makes use of the two-hybrid system (De Jaeger *et al.*, 2000). Here, the genes encoding the antigen and the scFv fragment are cloned in two-hybrid vectors and transformed in a suitable yeast strain. ScFv-antigen interaction can be analyzed by measuring yeast growth on specialized media. Because in this *in vivo* system, the protein interactions occur in the reducing environment of the cytosol and the nucleus, it is useful to identify candidate scFv fragments for intracellular antibody applications. In the long run, stable scFv scaffolds, such as those isolated by De Jaeger *et al.* (1999) and Tavladoraki *et al.* (1999), could represent a suitable framework for engineering recombinant antibodies to be targeted to the cytosol.

Promising alternatives are fragments derived from the heavy-chain antibodies of Camelidae. Besides conventional four-chain antibodies, the Camelidae produce antibodies devoid of light chains (Hamers-Casterman *et al.*, 1993). Single-domain antibodies (VHH) derived from the heavy-chain antibodies are the smallest antigen recognition units. A camel VHH anti-tetanus toxoid gene has been expressed in *Nicotiana tabacum* and targeted to the chloroplasts

by using an N-terminal chloroplast-targeting peptide. Functional VHH protein accumulated to levels up to 0.1% of the TSP in total leaf extracts. However, no VHH proteins were found in chloroplast extracts (Vũ, 1999). Interestingly, these heavy-chain antibodies are a unique source of inhibitory antibodies (Lauwereys *et al.*, 1998). Due to the peculiar topology of their antigen-binding site, many heavy-chain antibodies bind to clefts or cavities of enzymes, such as substrate-binding sites. Therefore, VHH domains seem attractive antibody fragments for immunomodulation of enzyme activity by competitive inhibition.

Concluding remarks

The plantibody approach is one of the most recent innovations in the field of molecular techniques for the analysis and manipulation of plant cellular pathways and pathogen infection. Full inactivation of genes by using antisense, co-suppression, and dominant-negative mutants is rarely achieved and can create problems when low levels of mRNA and/or protein are sufficient for normal gene expression (Bourque, 1995). Therefore, the plantibodies can be used as an alternative to, or in combination with, the above-mentioned reverse genetics techniques. Moreover, we believe that the plantibody approach has several unique features. First, the activity of non-protein agents can be targeted. Second, resistance against plant-pathogenic microbes can be obtained, and there are good indications that this approach can also be applied to more complex pathogens, such as nematodes. So far, direct attack of pathogens with antisense or co-suppression has only been feasible when the genome of the pathogen is inserted into the plant host cell, as in the case of viruses. Third, antibody production could offer a more fine-tuned approach to modulate protein activity and, as such, to analyze gene activity in more detail. Specific epitopes, instead of the whole proteins, could be targeted. Moreover, applying antibodies with the same target specificity but with different binding affinities might permit different levels of epitope blocking. As proteins often interact with more than one other protein, it is possible that blocking a single epitope may leave other protein interactions intact, resulting in less pleiotropic effects than a total gene knock-out. In this way, the functional study of epitopes instead of complete proteins becomes feasible.

Acknowledgements

We thank all the members of the ‘plantibody’ consortium, and especially Arjen Schots, for all the stimulating discussions regarding this subject, Martine De Cock for lay-out, and Rebecca Verbanck for artwork. The laboratory of the authors was financially supported by a grant from the European Commission (FAIR1-CT95-0905). G.D.J. and E.F. are indebted to the Vlaams Instituut voor de Bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie for a postdoctoral and predoctoral fellowship, respectively.

References

Artsaenko, O., Peisker, M., zur Nieden, U., Fiedler, U., Weiler, E.W., Müntz, K. and Conrad, U. 1995. Expression of a single-chain Fv antibody against abscisic acid creates a wilty phenotype in transgenic tobacco. Plant J. 8: 745–750.

Artsaenko, O., Phillips, J., Fiedler, U., Peisker, M. and Conrad, U. 1999. Intracellular immunomodulation in plants - a new tool for the investigation of phytohormones. In: K. Harper and A. Ziegler (Eds.), Recombinant Antibodies: Applications in Plant Science and Plant Pathology, Taylor & Francis, London, pp. 145–156.

Baum, T.J., Hiatt, A., Parrott, W.A., Pratt, L.H. and Hussey, R.S. 1996. Expression in tobacco of a functional monoclonal antibody specific to stylet secretions of the root-knot nematode. Mol. Plant-Microbe Interact. 9: 382–387.

Ben-Yakir, D. and Shochat, C. 1996. The fate of immunoglobulin G fed to larvae of *Ostrinia nubilalis*. Entomol. Exp. Appl. 81: 1–5.

Bourque, J.E. 1995. Antisense strategies for genetic manipulations in plants. Plant Sci. 105: 125–149.

Chen, Y.D. and Chen, T.A. 1998. Expression of engineered antibodies in plants: a possible tool for spiroplasma and phytoplasma disease control. Phytopathology 88: 1367–1371.

de Haard, H.J., van Neer, N., Reurs, A., Hufton, S.E., Roovers, R.C., Henderikx, P., de Bruïne, A.P., Arends, J.-W. and Hoogenboom, H.R. 1999. A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. J. Biol. Chem. 274: 18218–18230.

De Jaeger, G., Buys, E., Eeckhout, D., Bruyns, A.-M., De Neve, M., De Wilde, C., Gerats, T., Van Montagu, M., Fischer, R. and Depicker, A. 1997. Use of phage display for isolation and characterization of single-chain variable fragments against dihydroflavonol 4-reductase from *Petunia hybrida*. FEBS Lett. 403: 116–122.

De Jaeger, G., Buys, E., Eeckhout, D., De Wilde, C., Jacobs, A., Kapila, J., Angenon, G., Van Montagu, M., Gerats, T. and Depicker, A. 1999. High level accumulation of single-chain variable fragments in the cytosol of transgenic *Petunia hybrida*. Eur. J. Biochem. 259: 426–434.

De Jaeger, G., Fiers, E., Eeckhout, D. and Depicker, A. 2000. Analysis of the interaction between single-chain variable fragments and their antigen in a reducing intracellular environment using the two-hybrid system. FEBS Lett. 467: 316–320.

De Wilde, C., Peeters, K., Van Montagu, M. and Depicker, A. 1998. Transient expression of IgG antibodies and antibody fragments in intact leaf tissue of *Nicotiana tabacum*. Meded.. Fac. Landbouwwet. Univ. Gent 63/4b: 1703–1706.

De Wilde, C., De Jaeger, G., De Neve, M., Van Montagu, M. and Depicker, A. 1999. Production of antibodies in transgenic plants - a general introduction. In: K. Harper and A. Ziegler (Eds.) Recombinant Antibodies: Applications in Plant Science and Plant Pathology, Taylor & Francis, London, pp. 113–127.

East, I.J., Fitzgerald, C.J., Pearson, R.D., Donaldson, R.A., Vuocolo, T., Cadogan, L.C., Tellam, R.L. and Eisemann, C.H. 1993. *Lucilla cuprina*: inhibition of larval growth induced by immunization of host sheep with extracts of larval peritrophic membrane. Int. J. Parasitol. 23: 221–229.

Fecker, L.F. and Koenig, R. 1999. Engineering of Beet necrotic yellow vein virus (BNYVV) resistance in *Nicotiana benthamiana*. In: K. Harper and A. Ziegler (Eds.) Recombinant Antibodies: Applications in Plant Science and Plant Pathology, Taylor & Francis, London, pp. 157–170.

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. Arch. Virol. 142: 1857–1863.

Fischer, R., Liao, Y.-C., Hoffmann, K., Schillberg, S. and Emans, N. 1999. Molecular farming of recombinant antibodies in plants. Biol. Chem. 380: 825–839.

Griffiths, A.D., Williams, S.C., Hartley, O., Tomlinson, I.M., Waterhouse, P., Crosby, W.L., Kontermann, R.E., Jones, P.T., Low, N.M., Allison, T.J., Prospero, T.D., Hoogenboom, H.R., Nissim, A., Cox, J.P.L., Harrison, J.L., Zaccolo, M., Gherardi, E. and Winter, G. 1994. Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J. 13: 3245–3260.

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

Hanes, J. and Plückthun, A. 1997. *In vitro* selection and evolution of functional proteins by using ribosome display. Proc. Natl. Acad. Sci. USA 94: 4937–4942.

Huits, H.S.M., Gerats, A.G.M., Kreike, M.M., Mol, J.N.M. and Koes, R.E. 1994. Genetic control of dihydroflavonol 4-reductase gene expression in *Petunia hybrida*. Plant J. 6: 295–310.

Kapila, J., De Rycke, R., Van Montagu, M. and Angenon, G. 1997. An *Agrobacterium*-mediated transient gene expression system for intact leaves. Plant Sci. 122: 101–108.

Lauwereys, M., Ghahroudi, M.A., Desmyter, A., Kinne, J., Hölzer, W., De Genst, E., Wyns, L. and Muyldermans, S. 1998. Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. EMBO J. 17: 3512–3520.

Le Gall, F., Bové, J.-M. and Garnier, M. 1998. Engineering of a single-chain variable-fragment (scFv) antibody specific for the stolbur phytoplasma (mollicute) and its expression in *Escherichia coli* and tobacco plants. Appl. Environ. Microbiol. 64: 4566–4572.

Lehane, M.J. 1996. Digestion and fate of the vertebrate bloodmeal in insects. In: S.K. Wikel (Ed.) The Immunology of Host-Ectoparasitic Arthropod Relationships, CAB International, Wallingford, UK, pp. 131–149.

Lener, M., Horn, I.R., Cardinale, A., Messina, S., Nielsen, U.B., Rybak, S.M., Hoogenboom, H.R., Cattaneo, A. and Biocca, S. 2000. Diverting a protein from its cellular location by intracellular antibodies. The case of p21Ras. Eur. J. Biochem. 267: 1196–1205.

Marasco, W.A. 1995. Intracellular antibodies (intrabodies) as research reagents and therapeutic molecules for gene therapy. Immunotechnology 1: 1–19.

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

Phillips, J., Artsaenko, O., Fiedler, U., Horstmann, C., Mock, H.-P., Müntz, K. and Conrad, U. 1997. Seed-specific immunomodulation of abscisic acid activity induces a developmental switch. EMBO J. 16: 4489–4496.

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. Biochem. Biophys. Res. Commun. 220: 255–263.

Sblattero, D. and Bradbury, A. 2000. Exploiting recombination in single bacteria to make large phage antibody libraries. Nature Biotechnol. 18: 75–80.

Schouten, A. 1998. Plantibodies - Requirements for Expression and Subcellular Targeting. Ph.D. thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Schouten, A., Roosien, J., van Engelen, F.A., de Jong, G.A.M.I., 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 Mol. Biol. 30: 781–793.

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.

Sheets, M.D., Amersdorfer, P., Finnern, R., Sargent, P., Lindqvist, E., Schier, R., Hemingsen, G., Wong, C., Gerhart, J.C. and Marks, J.D. 1998. Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. Proc. Natl. Acad. Sci. USA 95: 6157–6162.

Shimada, N., Suzuki, Y., Nakajima, M., Conrad, U., Murofushi, N. and Yamaguchi, I. 1999. Expression of a functional single-chain antibody against $GA_{24/19}$ in transgenic tobacco. Biosci. Biotechnol. Biochem. 63: 779–783.

Smant, G., Stokkermans, J.P.W.G., Yan, Y.T., de Boer, 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. Proc. Natl. Acad. Sci. USA 95: 4906–4911.

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.

Tavladoraki, P., Girotti, A., Donini, M., Arias, F.J., Mancini, C., Morea, V., Chiaraluce, R., Consalvi, V. and Benvenuto, E. 1999. A single-chain antibody fragment is functionally expressed in the cytoplasm of both *Escherichia coli* and transgenic plants. Eur. J. Biochem. 262: 617–624.

Vũ, K.B. 1999. Llama Antibodies and Engineering of Single-Domain Antibody Fragments. Ph.D. thesis, Vrije Universiteit Brussel, Brussels, Belgium.

Vaughan, T.J., Williams, A.J., Pritchard, K., Osbourn, J.K., Pope, A.R., Earnshaw, J.C., McCafferty, J., Hodits, R.A., Wilton, J. and Johnson, K.S. 1996. Human antibodies with sub-nanomolar

affinities isolated from a large non-immunized phage display library. Nature Biotechnol. 14: 309–314.

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. Mol. Breed. 1: 39–50.

Wattad, C., Kobiler, D., Dinoor, A. and Prusky, D. 1997. Pectate lyase of *Colletotrichum gloeosporioides* attacking avocado fruits: cDNA cloning and involvement in pathogenicity. Physiol. Mol. Plant Pathol. 50: 197–212.

Wong, J.Y.M. and Opdebeeck, J.P. 1993. Immunity in vaccinated cattle exposed to experimental and natural infestations with *Boophilus microplus*. Int. J. Parasitol. 23: 689–692.

Zimmermann, S., Schillberg, S., Liao, Y.-C. and Fisher, R. 1998. Intracellular expression of TMV-specific single-chain Fv fragments leads to improved virus resistance in *Nicotiana tabacum*. Mol. Breed. 4: 369–379.