INVITED REVIEW

ANTIBODY-BASED PATHOGEN RESISTANCE IN PLANTS

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SUMMARY

Genetic engineering can be used to create plants with enhanced resistance to disease. Several strategies have been developed and tested, including expression of pathogen-derived sequences, the modification and overexpression of endogenous resistance genes, and the expression of heterologous resistance genes from diverse sources. Another promising approach is the expression of recombinant antibodies that bind to and neutralize essential components of the pathogen and therefore interfere with its infection cycle. The impact of this approach depends on appropriate target selection, careful antibody design, subcellular targeting of the recombinant antibodies and antibody accumulation levels and stability. This review describes the current status of antibody-based disease resistance in plants, focussing on optimization and the remaining obstacles to its widespread use.

Key words: genetic engineering, molecular biotechnology, protein targeting, recombinant antibodies, transgenic plants.

INTRODUCTION

Plant disease is responsible for significant losses of global crop production every year, and thus has a major impact on the world's agricultural productivity. Numerous strategies have been developed in attempts to minimize the losses caused by plant pathogens. Traditional approaches are based on the avoidance of sources of infection, vector management, modification of cultural practices, the use of resistant varieties obtained through conventional breeding, cross protection and chemical control. While these methods have been successful in some cases, indeed most crop varieties in use today incorporate some form of genetic resistance (Crute and Pink, 1996), they are time-consuming, and the pathogens or pests can adapt to existing resistance genes and/or pesticides within a short time. Therefore, despite the continual development of new resistant cultivars and pesticides, annual crop losses caused by plant pathogens, insect pests and weeds, have steadily increased to 42% worldwide, causing damage estimated at $500 billion in the process (Oerke *et al*., 1994), despite the continued release of new resistant cultivars and pesticides.

Furthermore some pesticides are being withdrawn from the market because of their undesirable effects on the environment. New strategies for disease control are therefore urgently required.

The development of novel control strategies for plant diseases is particularly important for pathogens that are difficult to control using existing methods. The creation of disease-resistant plant varieties requires an understanding of plant disease mechanisms, pathogen life cycles and pathogen-plant interactions. Once these principles are understood, it is possible to exploit the information to develop new approaches that provide broad-spectrum pathogen resistance in genetically modified plants.

In this review, we briefly describe the molecular approaches used to generate disease-resistant plants, focusing on antibody-based resistance, which involves the expression of cloned antibody genes that neutralise target pathogens by interfering with their life cycle (Schillberg *et al*., 2001). We discuss ways in which the efficiency and potency of antibody-based resistance can be improved by antibody engineering and by optimizing the expression levels and stability of recombinant antibodies *in planta*.

CONVENTIONAL PATHOGEN CONTROL APPROACHES

Advances in molecular biotechnology and plant transformation have facilitated the development of transgenic plants that are resistant to a number of different pathogens, including viruses, bacteria, fungi and invertebrates. Some of the different strategies that have been used are:

- The modification and transfer of natural resistance

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genes between species to create varieties resistant to one or more pathogens.
- The expression of heterologous resistance proteins from bacteria, fungi and animals to interfere with various stages of the pathogen life cycle.
- The expression of RNA or proteins from a viral pathogen (or a close relative) to provide pathogen-derived resistance.

Natural and engineered resistance genes. Plants use a number of pathogen-induced defence pathways to prevent or control disease, and together these constitute the general or innate immune system. The earliest defence response against pathogens is the hypersensitive response, which is considered to be the main step leading to systemic acquired resistance (SAR). The latter is the most intensively studied pathogen-induced defence response, and provides broad-spectrum resistance against not only the initial pathogen challenge, but also against subsequent infection by a variety of other viral, fungal and bacterial pathogens (Ryals *et al*., 1996; Delaney, 1997; Sticher *et al*., 1997).

The induction of this natural defence mechanism requires the specific recognition of the pathogen, and in many cases is based on matching resistance gene products from the plant (produced by the dominant resistance (R) genes) to the products of the pathogen avirulence (avr) genes (for recent reviews, see Dangl and Jones, 2001; Goldbach *et al*., 2003). A growing number of mostly dominant resistance genes have been cloned and analyzed, leading to a better understanding of natural defence systems in plants. This has also allowed such genes to be transferred between species, e.g. the tobacco *N* gene, which confers resistance to *Tobacco mosaic virus* (TMV), has been transferred to tomato establishing TMV resistance in this species (Whitham *et al*., 1996).

In other cases, pathogenesis-related (PR) proteins or defence peptides have been overexpressed, or plant metabolism has been modified to increase the production of antimicrobial compounds such as thionins, snakins, hevein- and knottin-like peptides, MBP1, IbAMP and so-called lipid transfer proteins (Broekaert *et al*., 1997; Epple *et al*., 1997; Garcia-Olmedo *et al*., 1998; Rommens and Kishore, 2000). Plant-derived ribosomal inactivating proteins have also been expressed in transgenic plants. These have a limited antifungal activity but also provide strong resistance against a broad range of plant viruses (Moon *et al*., 1997; Tumer *et al*., 1997). It is also possible to modify resistance genes for improved performance and reintroduce such genes into the original host species. For example, an engineered version of the rice cysteine proteinase inhibitor oryzacystatin has been expressed in transgenic rice to confer resistance to nematodes (Vain *et al.,* 1998).

The disadvantage of natural, plant-derived resistance genes is that the resistance conferred on the plant is often short-lived since the pathogen can adapt rapidly to overcome it. Although the correlation between the onset of SAR and the high-level expression of many PR genes is tightly regulated, transgenic plants expressing these PR genes display only modest resistance against pathogens (Alexander *et al*., 1993). This indicates that the concerted expression of several PR genes may be required to produce a robust defence against invading pathogens, or that other important resistance effectors have yet to be discovered. Alternative strategies for more durable resistance have been investigated. These include the expression of heterologous proteins with antiviral or antimicrobial activities that naturally occur in insects, plants, animals and humans. Such proteins include antiviral ribonucleases and mammalian 2',5' oligoadenylate synthetases (Watanabe *et al*., 1995; Ogawa *et al*., 1996), insect-derived lytic peptides for bacterial resistance (e.g. Jaynes *et al*., 1987, 1993; Huang *et al*., 1997) and human lysozyme and lactoferrin for resistance against bacteria and fungi (Mitra and Zhang, 1994; Nakajima *et al*., 1997). The expression of ribozymes has also been investigated as a strategy to produce virus-resistant plants (de Feyter *et al*., 1996; Kwon *et al*., 1997).

Pathogen-derived resistance. Pathogen-derived viral resistance is based on the expression of a pathogen-derived protein (protein-mediated resistance) or nucleic acid sequence (RNA-mediated resistance) to interfere with particular aspects of the pathogen life cycle. Although the concept of pathogen-derived viral resistance was introduced by Hamilton in 1980, the first practical demonstration came more than five years later, when Powell *et al*. (1986) created transgenic tobacco plants expressing the TMV coat protein gene and showed that the plants were resistant to TMV because of the expressed coat protein. Many virus-resistant transgenic have been produced subsequently by expressing different viral sequences (reviewed by Lomonossoff, 1995; Beachy, 1997, 1999; Bendahmane and Beachy, 1999) including some that have reached commercial status (see Tricoli *et al*., 1995). The most successful and widely applied strategy has been the expression of viral coat protein (CP) genes, thus achieving CP-mediated resistance.

Other successful approaches for achieving pathogen-derived resistance include the transformation of plants with a mutagenized movement protein (MP) or replicase subunit and with cDNA copies of symptom-suppressing satellites (Harrison *et al*., 1987; Anderson *et al*., 1992; Lapidot *et al*., 1993; Audy *et al*., 1994; Baulcombe, 1994; Palukaitis and Zaitlin, 1997). Although protein-mediated resistance strategies have been successful under greenhouse and field conditions, there are some safety concerns reflecting the possibility that *in planta* recombinations could produce wild type virus genomes or even completely novel viruses with unknown consequences (Aaziz and Tepfer, 1999; Borja *et al*., 1999; Rubio *et al*., 1999).

The most widely studied example of nucleic acid-mediated resistance is RNA-based post-transcriptional gene silencing (PTGS). In plants, PTGS is a natural antiviral defence system that monitors the cell for the appearance of aberrant RNAs, such as double stranded viral replication intermediates. The basis of RNA-mediated resistance is the sequence-specific degradation of viral transcripts caused by the appearance of small amounts of double stranded RNA homologous to the corresponding viral gene (reviewed by Hammond *et al.*, 2001). The same effect can be achieved by the expression of antisense RNA (Bourque, 1995) and satellite RNAs (Baulcombe *et al.*, 1986; Harrison *et al.*, 1987). The activity of at least one natural resistance gene has also been found to involve RNA-silencing (Covey *et al.*, 1997). The general model of virus- and transgene-induced PTGS in plants is reviewed by Goldbach *et al.* (2003). While the effects of protein-mediated resistance and PTGS can overlap, it is notable that PTGS requires transcription of the viral genome whereas protein-specific protective effects take place before the viral genome is expressed (reviewed by Bendahmane and Beachy, 1999).

Although the RNA-based PTGS fulfils the current high demands with respect to biosafety (since it excludes the production of functional viral genes or proteins), its applications are limited to the protection of plants against viruses that can be used to deliver the transgene, or to closely related isolates (Prins *et al.*, 1996; van der Boogaart *et al.*, 2001). Furthermore, many plant viruses produce suppressors of gene silencing that confer short-term resistance against plants using PTGS as an antiviral mechanism.

The expression of pathogen-specific recombinant antibodies has the potential to overcome the limitations of both natural or engineered resistance genes and pathogen-derived resistance. Before discussing how protective antibodies are expressed in plants, we briefly review the technology available for antibody production.

TECHNOLOGY FOR RECOMBINANT ANTIBODY PRODUCTION

Recombinant antibodies originated in the 1970s, with the advent of hybridoma technology for the production of monoclonal antibodies against any conceivable antigen (Koehler and Milstein, 1975). Traditional hybridoma technology is limited to the production of full size monoclonal antibodies of murine origin, but recombinant DNA methods now allow the production of derivatized molecules recognizing any target antigen and the fine-tuning of these recombinant antibodies to enhance their desirable properties (Winter *et al.*, 1994; Kipriyanov and Little, 1999). Thus it is possible to design and express full length recombinant antibodies and derivatives that are reduced in size, dissected into minimal binding fragments and rebuilt into multivalent, high-avidity reagents (Kipriyanov and Little, 1999; Tomlinson and Holliger, 2000; Humphreys and Glover, 2001). Such derivatives include single chain Fv (scFv) fragments, bispecific scFvs, diabodies, minibodies, Fab fragments and $F(ab')_2$ fragments (Fig. 1). Because the

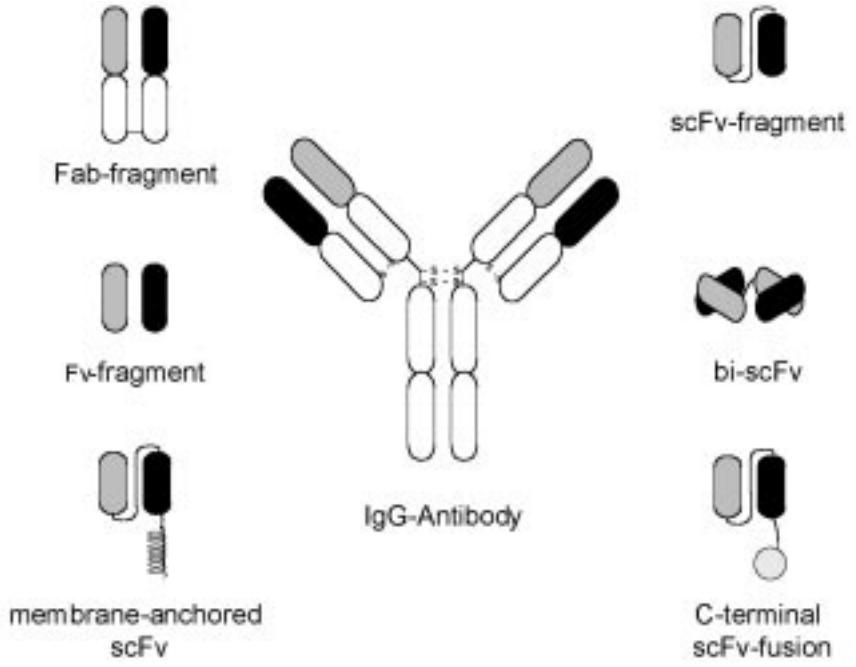

Fig. 1. Schematic representation of an IgG molecule and various derived recombinant antibody fragments produced in plants. Fv: fragment variable; scFv: single chain Fv.

Table 1. Antibody-based resistance to pathogens in transgenic plants.

Plant	Pathogen/Antigen	Antibody format	Localization	Biological effect	References
N. benthamiana	*Artichoke mottled crinkle virus* (coat protein)	scFv	Cytosol	Reduction of infection and delay in symptom development	Tavladoraki *et al.*, 1993
N. tabacum cv. Xanthi nc	*Tobacco mosaic virus* (coat protein)	full-size IgG_{2b}	Apoplast	70% reduction of local lesion number	Voss *et al.*, 1995
N. tabacum cv. Xanthi	*Meloidogyne incognita* (neamatode stylet secretions)	full-size antibody	Apoplast	No biological effect, probably due to mistargeting of the antibody	Baum *et al.*, 1996
N. benthamiana	*Beet necrotic yellow vein virus* (coat protein)	scFv	ER	Delay in symptom development	Fecker *et al.*, 1997
N. tabacum cv. Xanthi nc	*Tobacco mosaic virus* (coat protein)	scFv	Cytosol	>90% reduction of local lesion number, 11% of transgenic plants were fully resistant in systemic infection assays	Zimmerman *et al.*, 1998
N. tabacum	Stolbur Phytoplasma (major membrane protein)	scFv	Apoplast	Transgenic tobacco shoots grew free of symptoms	Le Gall *et al.*, 1998
Maize	*Spiroplasma kunkelii* (membrane protein)	scFv	Cytosol	No resistance	Chen and Chen, 1998
N. tabacum cv. Petite Havana SR1	*Tobacco mosaic virus* (coat protein)	scFv	Plasmalemma membrane surface	13% of transgenic plants were fully resistant in systemic infection assays	Schillberg *et al.*, 2000
N. tabacum cv. W38	Potato Virus strain Y and D, *Clover yellow vein virus* strain 300 (coat protein)	scFv	Cytosol, apoplast	Suppression of infection	Xiao *et al.*, 2000
N. tabacum cv. Samsun NN	*Tobacco mosaic virus* (coat protein)	scFv	Cytosol	100% reduction of virus infection	Bajrovic *et al.*, 2001

antigen-binding surfaces of these small antibody fragments are unaltered, they can provide the same binding specificity as intact, full-size antibodies. Also, the specificity and affinity of existing antibodies can be improved by grafting the complementary-determining regions (CDR) and by chain shuffling (Griffith, 1993; Hoogenboom and Winter, 1992; Marks *et al.*, 1992).

Traditionally, antibody cDNAs have been isolated from hybridoma cell lines. The standard procedure is to inject mice with the pathogen of interest, isolate B-lymphocytes and fuse these with immortalized myeloma cells under appropriate selective conditions to establish a hybridoma line. Hybridomas are then screened for their ability to produce antibodies that bind to the pathogen-derived antigen. Hybridoma technology allows the production of highly specific monoclonal antibodies, but the process is labour intensive and requires the use of animals, animal cell culture and expensive equipment. Many molecular biology laboratories have neither the facilities nor the experience to generate monoclonal antibodies in this manner. Importantly, hybridoma technology also does not allow the immediate and convenient isolation and cloning of immunoglobulin-encoding cDNAs.

Phage display is a powerful *in vitro* technique for the rapid selection of high affinity recombinant antibody fragments recognizing any protein of interest (Barbas *et al.*, 1991; Hoogenboom *et al.*, 1998). Antibody cDNAs are inserted into the phage genome so that they are fused in-frame with the phage coat protein gene. The fusion proteins are thus displayed on the surface of the mature phage resulting in a library of phage with antibodies presented on the surface and the corresponding genetic material contained within the particle. This linkage between genotype and phenotype allows enrichment of specific phages (Hoogenboom, 1997; Barbas, 1993). The phages are selected on immobilized targets by a simple *in vitro* selection procedure called biopanning in which phages that display relevant binding structures are retained, while non-adherent phages are washed away.

Phage display libraries can be derived from V-gene

repertoires of immunized (exposed to antigen) or naïve (not exposed to antigen) human or animal donors (Clackon *et al*., 1991; Marks *et al*., 1992). The phage display libraries from immunized donors are enriched for antibodies specific to the antigen of interest. Moreover, the antibodies that have undergone affinity maturation also have high affinity for the antigen. This advantage is offset by the need to make a specific phage display library for each antigen. The naïve library on the other hand allows antibodies recognizing a large panel of antigens to be selected, including self, nonimmunogenic and relatively toxic antigens without the need for immunization (Griffiths *et al*., 1993; Marks *et al*., 1992; Vaughan *et al*., 1996). Alternatively, synthetic or semisynthetic human antibody V-gene repertoires have been made by *in vitro* assembly of V gene segments and D/J fragments (de Kruif *et al*., 1995; Griffiths *et al*., 1993). Therefore, with the advent of phage display technology it has become possible to obtain antibodies specific to any antigen and in a very short time.

PRODUCTION OF ANTIBODIES IN PLANTS

The first plant-derived antibody, a full-length IgG-recognizing phosphonate ester, was produced in transgenic tobacco plant about 15 years ago (Hiatt *et al*., 1989; Düring *et al*., 1990). Since that time, various immunoglobulin classes have been expressed in plants including monotypic and chimeric IgG, IgM and IgA (Ma *et al*., 1994, 1995; Voss *et al*., 1995; De Wilde *et al*., 1996; Baum *et a*l., 1996). In addition to full size antibodies, various functional antibody derivatives have also been produced successfully in plants. These include Fab fragments (de Neve *et al*., 1993; De Wilde *et al*., 1996), scFvs (Owen *et al*., 1992; Firek *et al*., 1993; Tavladoraki *et al*., 1993; Artsaenko *et al*., 1995; Fiedler *et al*., 1997; Schouten *et al*., 1997), bispecific scFvs (Fischer *et al*., 1999) and membrane-anchored scFvs (Schillberg *et al*., 2000; Vine *et al*., 2001) (Fig. 1).

Antibody expression is achieved by inserting the cloned cDNA into a plant expression cassette comprising a strong promoter, control elements that enhance protein synthesis and signals that direct the recombinant protein to the appropriate intracellular compartment (Fischer and Emans, 2000). Comparative experiments, in which the same antibody has been targeted to different compartments, have shown that full-length antibodies accumulate to higher levels in the secretory pathway than in any other compartment (Schillberg *et al*., 1999, 2003). This is achieved through the inclusion of an N-terminal signal peptide, which may be of plant or mammalian origin and generally results in the antibodies being secreted to the apoplast, the intracellular space beneath the cell wall (e.g. see Voss *et al*., 1995). If a transmembrane anchor sequence is included, the antibody will be inserted into the plasma membrane (Schillberg *et al*., 2000; Vine *et al*., 2001). Antibody expression levels can be increased even further if the protein is retrieved to the endoplasmic reticulum (ER) lumen using an H/KDEL C-terminal tetrapeptide tag (Conrad and Fiedler, 1998). Accumulation levels in the ER are generally 2- to 10-fold greater than those of identical proteins lacking the KDEL signal. It is thought that the oxidizing environment of the ER, the lack of proteases and the abundance of molecular chaperones are important factors for correct protein folding and assembly. Additionally, protein glycosylation occurs only within the endomembrane system and this modification may in some cases contribute to the stability of full-length immunoglobulins. Folding and assembly problems related to the accumulation of full-length antibody format in

Table 2. Pathogen-specific antibodies expressed in plants (biological activity not tested).

Plant	Pathogen/Antigen	Antibody format	Localization	References
N. tabacum cv. Samsun NN	*Botrytis cinerea*-produced cutinase	Full-size	Apoplast	Van Engelen *et al*., 1994
Tobacco protoplasts	*Meloidogyne incognita* (nematode salivary secretions)	scFv	Cytosol, apoplast, ER	Rosso *et al*., 1996
N. benthamiana	*Tomato spotted wilt virus* (glycoprotein G1)	scFv	Apoplast	Franconi *et al*., 1999
N. tabacum cv. Petite Havana SR1, *N. tabacum* cv. Xanthi nc	*Tobacco mosaic virus* (coat protein monomer)	Full-size, scFv	Apoplast, cytosol	Schillberg *et al*., 1999
N. tabacum cv. Petite Havana SR1	*Tobacco mosaic virus* (coat protein, coat protein monomer)	Bispecific scFv	Cytosol, apoplast, ER	Fischer *et al*., 1999b
A. thaliana	*Fusarium*-produced mycotoxin (zearalenone)	scFv	Apoplast	Yuan *et al*., 2000
N. tabacum	*Citrus tristeza virus* (coat protein)	scFv	Cytosol?	Galeffi *et al*., 2002

non-secretory compartments can be circumvented by using single chain Fv fragments (scFvs) containing the complete antigen-binding site on one polypeptide chain. In scFv fragments (~30 kDa), the V_H and V_L domains are covalently linked by a flexible peptide linker (usually 15-20 amino acids long, e.g. of the sequence $(Gly_4Ser)_3$, or the 218 linker) (Whitlow *et al.*, 1993). Functional, single-chain antibodies have been expressed in the plant cytosol (Owen *et al.*, 1992; Tavladoraki *et al.*, 1993; Zimmermann *et al.*, 1998) showing that cytosolic scFv fragments could be used for intracellular 'imunization' in non-secretory compartments (Benvenuto and Tavladoraki, 1995). However, pathogen-specific antibodies must be targeted to the plant cell compartment where the pathogen is most vulnerable. This means that virus-specific antibodies are more likely to be effective in the cytosol whereas targeting to the apoplast would be more appropriate for defence against fungal pathogens.

ANTIBODY-BASED RESISTANCE - HISTORY AND RECENT PROGRESS

Resistance against viruses. The feasibility of antibody-based resistance has been demonstrated for plant viruses, and its application to other plant pathogens is becoming more established (Tables 1 and 2). The basis of antibody-based resistance is the neutralization of invading pathogens through interactions between high-affinity antibodies and critical pathogen proteins, thus preventing pathogen entry, replication and systemic spread. The first successful application of antibody-based resistance was reported by Tavladoraki *et al.* (1993). A scFv fragment specific for an isolate of *Artichoke mottled crinkle virus* (AMCV) was constructed from the parent monoclonal antibody selected from a panel raised against AMCV. The scFv was expressed in the plant cytosol and both transgenic protoplasts and plants were shown to be resistant to AMCV challenge.

Subsequently, full-length TMV-specific monoclonal antibodies were shown to protect tobacco plants against TMV infection when expressed in the apoplast (Voss *et al.*, 1995). In this study, the degree of protection correlated with the expression level. When the antibody reached 0.23% of total soluble protein, TMV lesions were reduced by 70%. At 0.4% TSP, the lesions were reduced by almost 95%. Interestingly, cytosolic expression of a scFv fragment derived from this antibody also conferred resistance, even though the level of recombinant antibody accumulating in the plants was approximately 20,000-fold lower than in plants expressing full-length immunoglobulins (Zimmermann *et al.*, 1998). This study showed that even very small amounts of cytosolic antibody were able to neutralize invading virions. Reduction in local lesions was greater than 90% and at least 11% of transgenic plants were protected against systemic TMV infections.

Although the cytosol appears to be the most suitable site for anti-viral antibodies, the secretory pathway has been investigated as an alternative for scFv accumulation (Fecker *et al.*, 1997; Xiao *et al.*, 2000). Fecker *et al.* (1997) expressed a scFv fragment against an isolate of *Beet necrotic yellow vein virus* (BNYVV) in *Nicotiana benthamiana* and used a KDEL signal to make the antibody accumulate in the ER lumen. They reported delayed onset of disease symptoms when plants were challenged with the virus. More recently, we have developed a novel expression system in which a scFv antibody is targeted to the plasma membrane of tobacco cells by fusion to a mammalian transmembrane domain (Schillberg *et al.*, 2000). The membrane-anchored scFv, with the antigen-binding domain exposed to the apoplast, provided strong resistance against TMV.

Thus far, antibodies expressed in virus-resistant transgenic plants have been raised solely against viral coat proteins, which are the most likely to mutate to overcome this new form of immunity. The effectiveness of antibody-based viral resistance may be increased in the future if antibodies are targeted against viral proteins crucial for replication, movement and transmission. These proteins are far more structurally constrained than coat proteins, and offer the best hope for durable resistance against viral diseases. We have produced tobacco plants expressing a scFv fragment specific for the movement protein of an isolate of *Tomato spotted wilt virus* (TSWV). Ten anti-NS_M scFvs were isolated by phage display, characterized for binding activity by expression in *E. coli*, and expressed in the cytosol of transgenic plants. The antibodies were expressed at high levels (0.1-8% of TSP) and significantly delayed the onset of disease symptoms when the plants were challenged with the pathogen (Zhang *et al.*, unpublished data).

Resistance against bacteria and phytoplasma. Le Gall *et al.* (1998) showed that antibody-based resistance is also useful against bacterial pathogens. These investigators provided a potential strategy to control phytoplasma diseases by expressing a scFv specific for the stolbur phytoplasma major membrane protein. Stolbur phytoplasma are strictly limited to the sieve tubes within the phloem tissues. Therefore phytoplasma-specific scFvs were directed through the secretory pathway. Transgenic tobacco shoots expressing anti-phytoplasma scFvs were top-grafted onto tobacco plants heavily infected with phytoplasma. The shoots grew free of symptoms while non-transgenic grafted tobacco shoots showed severe disease symptoms.

In contrast, cytosolic expression of a scFv specific for corn stunt spiroplasma (CSS) in maize did not confer resistance to the pathogen (Chen and Chen, 1998). It

would be interesting to repeat this study using antibodies directed to the secretory pathway in phloem cells since CSS are also restricted to the sieve tubes.

Resistance against fungi. Fungal pathogens are the most challenging target for antibody-based resistance because they affect crops in two ways: by destroying plants and seeds in the field and by contaminating the harvested crop with fungal toxins. During infection, invasive mycelia spread throughout the host plant, secreting enzymes and toxins that are essential for pathogenesis and parasitization. These proteins and toxins are suitable targets for recombinant antibodies, and if effective antibody-based strategies could be developed then environmental pollution caused by the extensive use of fungicides could be avoided.

Thus far, antibodies have been raised against a number of fungal antigens including conidia proteins, secreted proteins and other compounds, cell wall fragments and cell surface antigens of mycelia (Pain *et al*., 1992; Robert *et al*., 1993; Goebel *et al*., 1995; Murdoch *et al*., 1998). The application of antibodies to prevent fungal infection in plants has succeeded *in vitro* but there has yet to be a demonstration of effective protection in transgenic plants. The *in vitro* studies showed that the development of disease symptoms in avocado, mango and banana infected with *Colletotrichum gloeosporioides* was inhibited if the inoculum was first mixed with polyclonal antibodies specific for the fungal pectate lyase (Wattad *et al*., 1997).

An attractive strategy is the use of recombinant antibody fusion proteins expressed in transgenic plants as shuttles to deliver anti-fungal polypeptides to the fungal cell surface, where they would attack and kill the invading hyphae. To prove this hypothesis we have generated scFvs against *Fusarium* surface proteins and expressed them as fusions with anti-fungal polypeptides in *Arabidopsis.* Bioassays demonstrated that the recombinant antibody fusion proteins conferred strong resistance to the fungal pathogen, and that the effect was stronger than either the antibody or the anti-fungal peptide expressed on its own (Peschen *et al*., unpublished data).

Engineering nematode resistance. Nematode-induced diseases are another important target for antibody-based resistance. However, attempts to inhibit nematode parasitism of tobacco roots through antibody expression have not been successful thus far. Full-length immunoglobulins raised against nematode stylet secretions had no effect on a root-knot nematode parasite when expressed in transgenic plants (Baum *et al*., 1996). The targeting of the antibody was probably an important factor in the above case as the antibody was directed to the apoplast, where it would be unable to inactivate the stylet secretions injected into cells. A scFv fragment of corresponding specificity expressed in the cytosol would probably be more effective.

The identification of new target antigens could also improve the impact of antibody-based nematode resistance. One suitable candidate is the cellulase expressed by invading root-knot nematode larvae. This enzyme is thought to play an important role during intracellular migration of the larvae through the root cortex to the vascular cylinder. A number of cellulases have been cloned from the root-knot nematode *Meloidogyne incognita* (Smant *et al*., 1998; Rosso *et al*., 1999). Furthermore, expression of recombinant antibodies under the control of promoters induced by nematode invasion (Favery *et al*., 1998) will allow pathogen-specific antibody production at the exact time and location where the pathogen is most vulnerable.

OPTIMISATION OF ANTIBODY-BASED RESISTANCE

The success of antibody-based resistance depends on three major factors:

- The selection of antibodies that are able to block a crucial step in the pathogen multiplication or transmission cycle, in combination with careful selection of the target molecule, appropriate design of the recombinant antibody format and selection procedures.
- High levels of recombinant antibody accumulation, reflecting the design of the expression construct.
- Functionality of the antibody in the plant cell compartment where the pathogen life cycle is to be interrupted, reflecting the intrinsic properties of the recombinant antibody.

A schematic overview of the antibody-based resistance strategy is presented in Fig. 2. A prerequisite of the antibody-based approach is the identification of a suitable target antigen. Progress in the understanding of plant disease mechanisms has identified many proteins critical to pathogen infection, development and spread. The identification of conserved domains crucial for pathogenesis, such as replicase active site, could improve the effectiveness of this strategy, providing broad-spectrum and long lasting protection, despite the rapid evolution of counter-resistance in some pathogens (especially RNA viruses). Moreover, in the case of viral pathogens, the production of high-affinity antibodies specific to domains involved in the stability of the virus capsid, could impair virus assembly and disassembly, and could neutralize the virus in the early stages of the infection cycle when it is at its lowest abundance (for a review see Benvenuto and Tavladoraki, 1995).

A complete knowledge of the pathogen infection cycle is required so that recombinant antibodies can be targeted to the appropriate subcellular site where the pathogen is retained. For example, since most steps involved in viral infection take place in the plant cell cy-

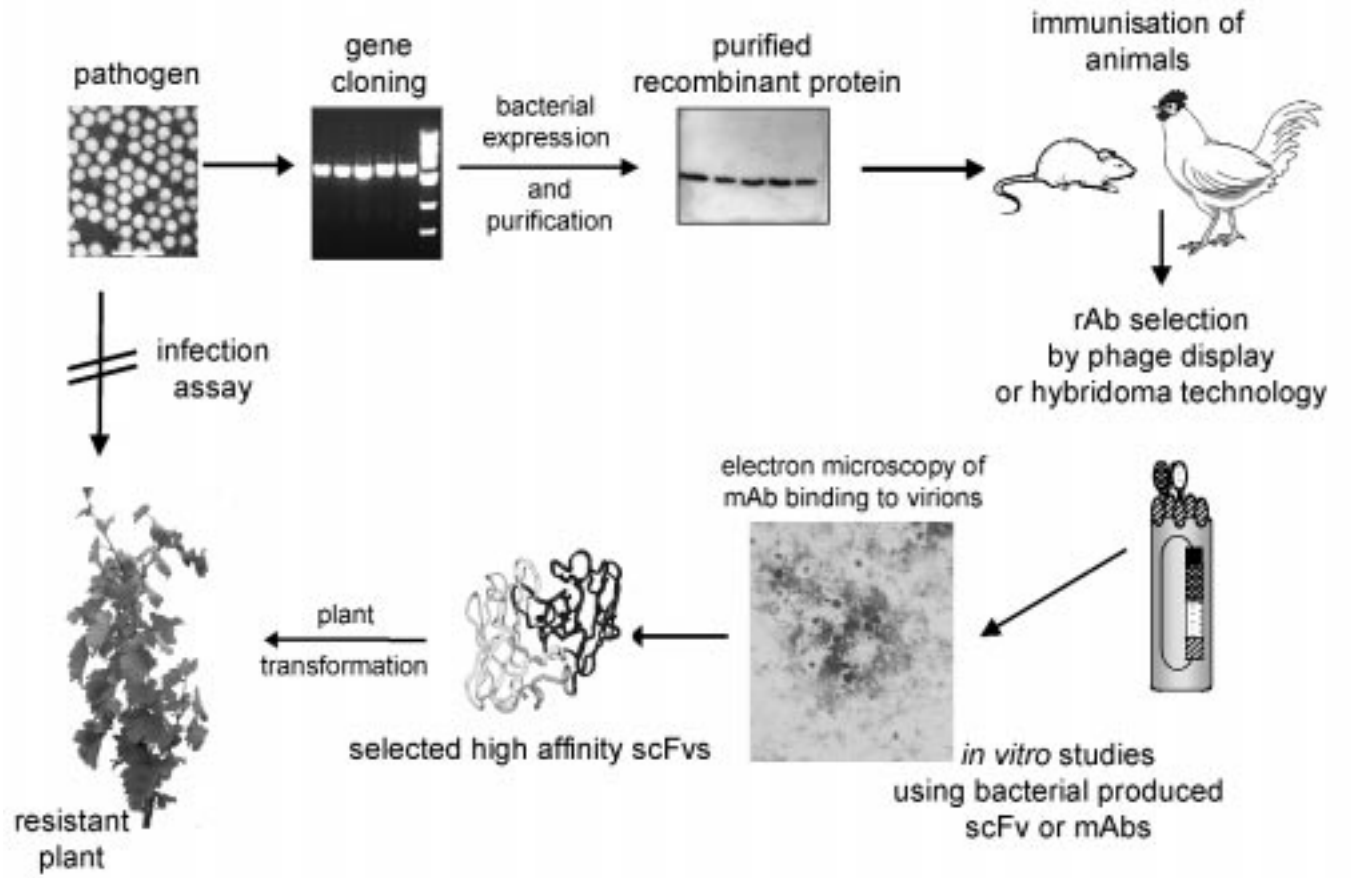

Fig. 2. Schematic overview of the antibody-based resistance strategy, using the overall strategy for the generation of transgenic grapevine plants producing virus-specific scFvs as an example.

tosol, expression of pathogen-specific recombinant antibodies in this compartment is the most appropriate strategy. The small molecular size of the scFv fragment, its unique and specific antigen binding capacity and the lack of specific folding or assembly requirements in the reducing environment of the plant cell cytosol makes this recombinant antibody format very attractive in antibody-based resistance. Furthermore, the small scFv molecule may be able to reach sites on the target that are sterically inaccessible to full-sized immunoglobulins and for a multimeric target, scFv molecules may bind to more sites than would the parental antibody (Benvenuto and Tavladoraki, 1995). In addition, the natural tendency of scFv fragments to dimerize, means that, scFvs recognizing different pathogen molecules could be combined to form bivalent or bispecific diabodies and triabodies to engineer pyramided resistance against individual pathogens. This type of resistance would be more difficult for the pathogen to break, because simultaneous adaptation would be required in multiple genes. The same strategy could be used to generate plant lines resistant to multiple pathogens.

The expression of scFv fragments in the cytosol provides adequate and in some cases better protection against viruses than high-level expression in the secretory pathway. Similarly, antibodies raised against nematode stylet secretions are ineffective when targeted to the apoplast, probably because the secretions are injected directly into the cytosol. Although many scFvs accumulate to low or undetectable levels in the cytosol (Owen *et al.*, 1992; Tavladoraki *et al.*, 1993; Schouten *et al.*, 1997; Zimmermann *et al.*, 1998), there are exceptional cases in which high-level expression (up to 1.0% TSP) has been achieved, indicating that stability is also dependent on intrinsic properties of the antibody itself (De Jaeger *et al.*, 1998). Cytosolic antibody expression may be further enhanced in the future by the identification of intrinsic structural features or fusion protein partners that stabilize the antibodies (Spiegel *et al.*, 1999; Worn *et al.*, 2000).

Antibody-based resistance can only succeed if the antibody and the target pathogen come into contact, so data on expression levels in different sub-cellular compartments is useful for evaluating the likely success of a chosen expression strategy. Previous studies have indicated that viral infection can be blocked in the cytosol, which would be expected because most steps of viral replication cycle take place there (Tavladoraki *et al.*, 1993; Zim-

mermann *et al*., 1998). However, we have recently described a novel method for protecting plant cells from viral infection that does not require cytosolic expression. Anti-viral antibodies were fused to a mammalian transmembrane domain and then expressed at the plasma membrane, facing the apoplast (Schillberg *et al*., 2000). These membrane-displayed antibodies provide at least the same level of resistance as those expressed as soluble proteins in the cytosol. The surface-displayed antibodies are produced at high levels and we speculate that they act to shield the plant from viral infection. The actual mechanism of the resistance is still under investigation, but painting the plant cells with a protective antibody might be also useful to inhibit bacterial or fungal pathogens.

PERSPECTIVE

The flexibility and specificity of antibodies is a well-known aspect of the vertebrate immune system and the use of antibodies to generate disease resistant plants is therefore an attractive approach. By exploiting antibody engineering and phage display (Winter and Milstein, 1991; Winter *et al*., 1994), it is possible to isolate antibodies that bind with high specificity to crucial proteins for pathogenesis resulting in a level of immunity or resistance to the pathogen. This means that almost any pathogen structure could be targeted by recombinant antibodies expressed in plants. Using an antibody against a critical conserved epitope may lead to long-lasting protection. An attractive property of the antibody-based resistance strategy is that multiple antibodies with different target specificities can be expressed in a single plant to engineer 'pyramided' resistance against individual or multiple pathogens.

The potential of this method is limited only by our understanding of plant-pathogen interactions. As our knowledge increases, and more targets are identified through genomic and proteomic approaches, antibodies can be designed and expression techniques tailored to suit each pathogen's profile. The integration of transgenic and antibody-based approaches with classical resistance breeding offers a potentially chemical-free and environmentally friendly solution for controlling plant disease.

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