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Plantibodies: A Novel Strategy to Create Pathogen-Resistant Plants

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Introduction

Under current control measures, plant pathogens, such as viruses, bacteria, fungi, and nematodes account for major losses in crop revenues worldwide that amount to hundreds of billions of dollars every year. In modern agriculture, breeding resistant plant cultivars is combined with chemical crop treatment. However, conventional breeding methods are limited by the scarcity of resistant germ plasm in nature and the rapid evolution of pathogens that break down natural resistance genes, thus, over time, defeating chemical treatments. Moreover, the chemical control of pathogens is expensive, labour-intensive, and portends health and environmental risks. Alternatives to classical and chemical crop protection methods are therefore required to circumvent the costs and risks of controlling pathogens in the field. Advances in the development of plant transformation and regeneration technologies, and in the understanding of the molecular mechanism underlying host–pathogen interactions, have provided alternative strategies for pathogen control through genetic engineering of crop plants.

Antibodies, or immunoglobulins, are defence molecules produced by all vertebrates. They recognize and bind to pathogen-specific antigens and thus help to eliminate pathogens from the body. Monoclonal antibodies produced by the hybridoma technique (Kohler and Milstein, 1975) display defined specificity and affinity for antigens. However, monoclonal antibodies are expensive to produce and

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Abbreviations: AFP, anti-fungal peptide or protein; AMCV, artichoke mottled crinkle virus; biscFv, bispecific single-chain variable fragment; CDR, complementary domain region; EDTA, ethylene diamine tetra-acetic acid; ER, endoplasmic reticulum; Fab, fragment antigen-binding; IgG, immunoglobulin G; MAb, monoclonal antibody; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; scFv, single-chain variable fragment; TMV, tobacco mosaic virus; TSP, total soluble proteins.

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maintain because specialized cell cultures and costly low-temperature storage facilities are required. In addition, some pathogens are difficult to obtain in a pure preparation, or are not amenable to antibody production. However, rapid progress in molecular immunology, combined with the invention of the polymerase chain reaction, has allowed fragments of antibody genes to be cloned and expressed in bacterial systems. By advanced technologies such as phage display or ribosome display, antibody fragments specific for particular antigens could be isolated *in vitro* from libraries containing diverse repertoires of antibody V-genes. These technical advances in recombinant antibody production have been applied widely to research in the plant sciences and biotechnology.

Plant-produced antibodies, namely *plantibodies*, were first demonstrated by Hiatt and colleagues and Duering and colleagues (Hiatt *et al.*, 1989; Duering *et al.*, 1990). These researchers demonstrated that plants can express and assemble functionally active antibodies, thus opening a new era in plant biology research. Since then, the technology of expressing antibodies in plants has advanced rapidly with a view to their utilization for therapeutic, diagnostic, and agricultural purposes (Schillberg *et al.*, 2001; Ma *et al.*, 2003). Many different forms of recombinant antibodies have been functionally expressed in a diverse range of plants by using the same pathway as mammalian cells for the assembly of light and heavy chains involving similar signal peptides and successful folding and assembly (Hood *et al.*, 2002). Generally, it has been assumed that after assembly in the lumen of the ER in plants, antibodies migrate via the secretory pathway to the intercellular space. It has been shown that full-size antibodies can be transported across both plasma membrane and cell wall in cell suspension cultures and *in planta* (Fischer *et al.*, 1999a; Nicholson *et al.*, 2005). This is in spite of the fact that their large molecular masses apparently exceed the mass exclusion limit with regards to the porosity of the plasma membrane and cell wall.

Expression of plantibodies and their proper assembly and transport in plants have resulted in an increasing awareness that this technology could be utilized for neutralizing and blocking plant pathogens, and thus for generating resistant plants. In this review, we describe some of the recent advances in the technology for creating transgenic plants that are highly resistant to pathogenic viruses, bacteria, and fungi through plantibodies. These include the generation of recombinant scFv antibodies against coat proteins of viruses and membrane proteins of mollicutes, and the selection by phage display of scFv antibodies specific for the surface antigens of fungi. Further relevant considerations involve localization of the binding site of fungus-specific scFv antibodies revealed by confocal immunofluorescence labelling, and functional characterization of plantibodies.

We discuss first the methods developed for the isolation of recombinant antibodies with high specificity and affinity, which serve as the foundation for plantibody-based resistance. After characterization in bacteria, the transient assay of antibody expression in plants is considered to be necessary for this strategy. The subcellular targeting of the antibody is shown to be another important consideration for a high level of expression. Targeting antibodies to the site, which is essential for pathogen infection and growth, generates a significantly enhanced resistance in transgenic plants. A more important advance to be considered here is that a recombinant fungus-specific antibody inhibits fungal growth *in vitro* when fused to

AFP. Expression of the fusion proteins in transgenic *Arabidopsis thaliana* plants conferred significant protection against *Fusarium* fungi. These results demonstrate that antibodies and their fusion proteins are both effective and versatile tools for the protection of crop plants against pathogens.

Generation of recombinant antibodies

As an alternative approach to the isolation of MAbs, phage display and ribosome display are used to isolate simultaneously antibodies and the corresponding gene sequences, which bypasses hybridoma technology altogether. This is based on the fact that the difference in antigen-binding specificities between antibodies lies entirely within their variable regions (CDRs). Therefore, it is necessary only to isolate genes for the variable domains (V-genes), which can then be joined to constant regions by recombinant DNA techniques. Antibody V-gene sequences can be amplified by RT-PCR from mRNA of immunized animals, or synthesized directly based on the antibody sequences from databases to facilitate the construction of large libraries (Winter *et al.*, 1994; Sachdev *et al.*, 2004), which is in principle similar to conventional cDNA expression libraries consisting only of coding sequences. Using molecular biological methods, appropriate antigens (like probes for hybridization) are used to screen libraries and to isolate antibodies together with their coding sequences (target genes). After several cycles of selection, antibodies with the highest affinity can be isolated.

The development of these technologies has made antibody generation more widely feasible with any antigen of interest in a more cost-effective manner. Numerous recombinant antibodies have been generated with this approach and have revealed wide application across various fields, including the improvement of plant resistance.

PHAGE DISPLAY

Phage display was developed to mimic the key features of antibody generation by the immune system for isolating antibody specificities (Smith, 1985; Winter *et al.*, 1994; Hoogenboom *et al.*, 1998). Large libraries (10^9) containing diverse repertoires of antibody V-genes can be constructed on phagemids by the fusion of antibody variable region genes (V-genes) to genes encoding for phage coat proteins (usually gene III). Expression of the fusion product in an appropriate bacterial host (amber suppressor) results in the display of antibody-binding fragments at the surface of the phage. Each phage displays a single antibody fragment comprising the variable regions of the heavy (V_H) and light (V_L) chains that form the Fab domains of natural antibodies, which is called a single-chain variable fragment (scFv).

Antibodies with desired specificities can be isolated by affinity selection to an immobilized antigen. Because the scFv gene contained in the phagemid encodes for the scFv antibody displayed on the surface of a phage particle, phage display directly links phenotype (scFv antibody) and genotype (scFv gene), as shown in *Figure 13.1*. Thus, the nucleotide sequence encoding for scFv antibodies can be determined readily and can be manipulated. Usually, several rounds of selection, called *panning*, are carried out to isolate the highest affinity scFv antibodies. In

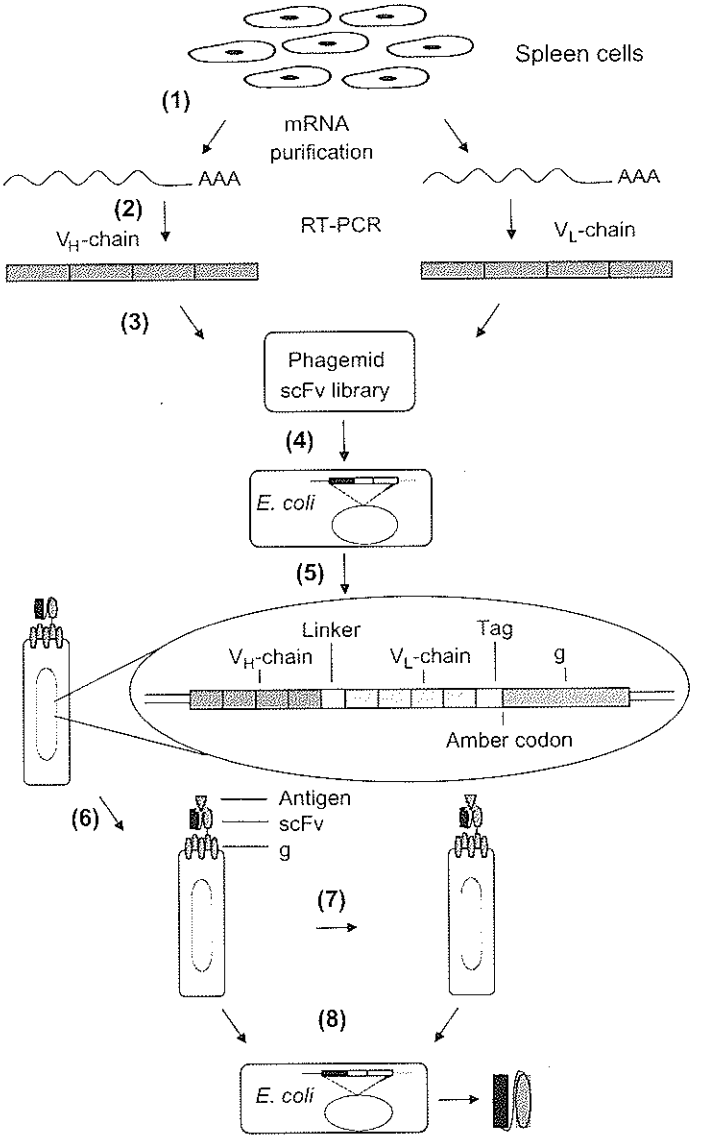

Figure 13.1. Generation of recombinant scFv antibodies by phage display. (1) Spleen cells from immunized animals are used for mRNA purification. (2) The mRNA is reverse-transcribed into cDNA and variable heavy chain sequences (V_H) and variable light chain sequences (V_L) are PCR-amplified. (3) V_H and V_L sequences are cloned into phagemid vector, such as pHEN4, to construct the scFv library. (4) The scFv library is used to transform *E. coli*. (5) scFv antibody is displayed on the surface of a phage particle. An amber codon is located at the end of tag sequence and the scFv molecule is expressed as a fusion to the minor coat protein encoded by gIII in a suppressor strain. The phage particle contains the phagemid encoding the scFv gene, thereby linking genotype and phenotype. (6) Antigen-driven affinity selection. Phage library is panned with target antigens. (7) Affinity maturation. Phages with high binding affinity are enriched by several rounds of panning. (8) Soluble antibody production. Phagemid from the selected phages is transformed into a non-suppressor strain to express soluble antibodies for further characterization of specificity and affinity.

addition, scFv antibodies also can be produced in a soluble form, unattached to the parent phage, when expressed in a non-suppressor bacterium.

Antibody phage display is the most contemporary technology used and has yielded hundreds of recombinant antibodies from mice (Winter *et al.*, 1994), humans (Sheets *et al.*, 1998), chicken (Andris-Widhopf *et al.*, 2000), and sheep (Charlton *et al.*, 2001), and also from rabbit (Foti *et al.*, 1998), camel (Gharoudi *et al.*, 1997), and cattle (O'Brien *et al.*, 1999).

Numerous cloning and mutagenesis strategies have been developed to create, preserve, and exploit maximal antibody diversity. As in the immune system, isolated scFv genes can be subjected further to mutation, and the mutants with more desirable characteristics then can be selected (Zahnd *et al.*, 2004). Phage display antibodies can be used in the same range of applications as their hybridoma counterparts, and moreover, can offer several advantages over hybridoma methods. Phage antibody generation is rapid and can be prepared in a few weeks using microgram levels of antigen (Hust and Duebel, 2004). Phage display can produce antibodies that are not readily prepared by hybridoma technology. These scFv antibodies can be manipulated genetically with ease for the construction and expression of new recombinant proteins, such as AFP–scFv fusions described below. In addition, phage display has been used also for the selection of other proteins with binding properties (Simon *et al.*, 2004).

RIBOSOME DISPLAY

Ribosome display was first described for peptides (Mattheakis *et al.*, 1994) and is an *in vitro* display technology that also links phenotype (the protein) directly to genotype (the mRNA). This technology has been developed based on two important findings: (1) a cell-free system, such as rabbit reticulocyte, capable of producing functional scFv antibodies; and (2) the formation of stable ternary polypeptide–ribosome–mRNA complexes in cell-free systems in the absence of a stop codon. Therefore, this allows for the production of scFv antibodies in rabbit reticulocyte systems, along with capture of the encoding mRNA (Hanes and Plueckthun, 1997; He and Taussig, 1997). An scFv DNA library can be expressed *in vitro* using an *in vitro* transcription and translation system. After addition of target antigens in the systems, the scFv antibody–ribosome–mRNA complexes bind to their specific antigens, while non-specific complexes and library components are removed by extensive washing. The complexes are disassociated with EDTA to release enriched antigen-binding sequences, and mRNAs are isolated. Eluted mRNAs are reversely transcribed to cDNA and re-amplified by PCR. The PCR products obtained are then used for the next cycle of enrichment and bacterial expression for further characterization of antibodies. Ribosome display method and cycles are presented in *Figure 13.2*.

The advantages of ribosome display are that, in comparison to phage display, larger libraries consisting of 10^{12} to 10^{13} DNA molecules can be constructed without the transformation step that is crucial for the success of phage display, and the libraries can be further diversified by PCR during ribosome display (Lipovsek and Plueckthun, 2004; Osbourn *et al.*, 2005). A good example is that scFvs specific for the terminal proteins of hepatitis B virus DNA polymerase have been isolated

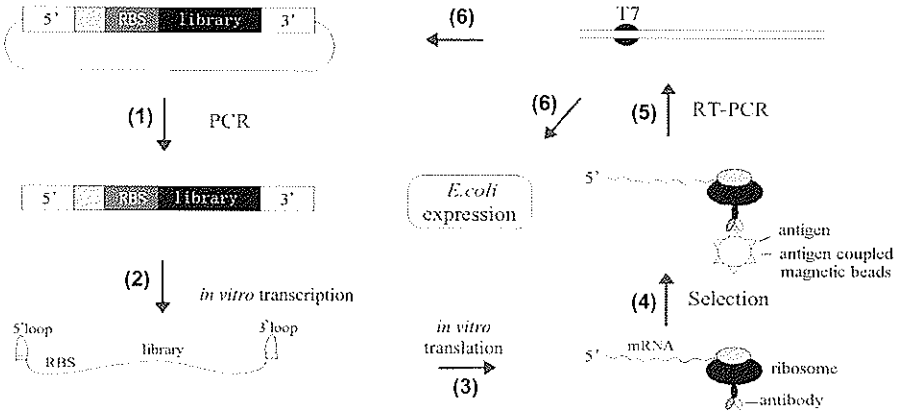

Figure 13.2. Generation of recombinant scFv antibodies by ribosome display. A ribosome display library is constructed on a ribosome display vector, such that gene sequences of library are flanked by a promoter (e.g. T7) and an RNA binding site (RBS) at an upstream region and a C-terminal spacer carrying no stop codon at 3' end connects the ribosome, and the folded protein is translated. (1) The library of interest is PCR-amplified with an upstream primer and a downstream primer annealed to the 5' and 3' regions, respectively. (2) *In vitro* transcription is performed with the PCR product generating mRNA carrying 5'-stem loop and 3'-stem loop that stabilizes the mRNA molecule. (3) The mRNA is used for *in vitro* translation with a rabbit reticulocyte lysate system, and the complexes of mRNA, ribosome, and nascent antibody are stably formed. (4) The complexes are subjected to selection by mixing the translation mix with magnetic beads coupled to antigen. The magnetic beads are collected and washed after incubation. (5) RT-PCR is carried out with the mRNA of the complexes. (6) The RT-PCR product can be cloned into the ribosome display vector for the next cycle and a prokaryotic expression vector for *E. coli* expression.

successfully by ribosome display, but phage display only generates scFv antibodies with weak binding activities (Lee *et al.*, 2004). Using this technology, scFv antibodies with high binding affinity to target antigens have been selected and evolved from mouse and human libraries (Hanes *et al.*, 2000; He and Taussing, 2005).

Antibody expression in plants

Monoclonal and recombinant antibodies have been produced in various expression systems, including plants and plant cell cultures, as intact antibodies, Fab, scFv, biscFv, or diabody. Plants have significant advantages in safety and costs over other expression systems, and they produce high levels of fully functional mammalian antibodies. Plant cells carry out many of the post-translational modifications required for optimal biological activity of antibodies and are attractive alternatives to bacteria because they produce fully functional antibodies with virtually identical specificity and affinity as monoclonal antibodies produced by hybridoma cell lines (Ko *et al.*, 2005). Antibodies can be expressed either transiently in plant leaves or stably in transgenic plants, depending on the applications required. Both expression systems in plants have been well established and widely exploited to produce antibodies for basic research and the pharmaceutical, agricultural, and biotechnology industries (Fischer *et al.*, 1999b; Ma *et al.*, 2005).

TRANSIENT EXPRESSION IN PLANTS

Transient gene expression in plants is a fast and flexible expression system for structural and functional characterization of recombinant antibodies without generation of transgenic plants. It can rapidly produce large amounts of antibodies. Transient expression in plants has advantages over the generation of stably transformed plants, and it is particularly suited for verifying that the gene product is functional and stable prior to moving on to large-scale production in transgenics. By contrast to stable plant transformation, which requires a considerable investment in time until analysis of the expressed antibodies, transient gene expression systems are rapid and are not influenced by positional effects that potentially bias gene expression levels in stably transformed plants (Kapila *et al.*, 1997; Vaquero *et al.*, 1999; Rodríguez *et al.*, 2005). Three different approaches have been used in transient expression systems: agroinfiltration with recombinant agrobacteria, infection with modified viral vector, and biolistic delivery of 'naked DNA'. Here, we focus on agroinfiltration used for transient expression of recombinant antibodies in intact leaves and whole plants due to its wide application and convenient combination of both transient assay and stable transformation using the same constructs. Expression with viral vectors requires a completely different setup of constructs, and the transfer of naked DNA into plants by particle bombardment is unsuitable for the expression of large amounts of foreign proteins.

Agroinfiltration utilizes the delivery of agrobacteria into intact leaf tissue by vacuum infiltration. As for generating transgenic plants, antibody genes are first cloned into binary vectors, which are transferred into suitable *Agrobacterium* strains. Recombinant agrobacterial cells are then used for leaf vacuum infiltration, and agrobacterial gene products effectively transfer DNA to the plant cell, where the antibody gene is expressed (*Figure 13.3*). Unlike generating transgenic plants, no selection to identify transformed cells is required since the leaf tissue is used only for transient protein production. It is believed generally that the transferred T-DNA does not get integrated into plant chromosome but is present in the nucleus, where it is transcribed, and this leads to transient expression of the recombinant antibody. This system can process a large number of samples at once and evaluate gene expression in a few days with large quantities. Expression levels observed in the transient assay are very consistent with those obtained with the transgenic approach. We routinely use this system to test expression of scFv antibodies derived from phage display or genes of interest, and to study their functions before moving to generating transgenic plants (Spiegel *et al.*, 1999; Li *et al.*, 2005).

STABLE EXPRESSION IN PLANTS

Stable expression in plants involves generating transgenic plants where the target gene is stably integrated into the plant genome and expressed throughout different generations. After initial functional and structural characterization of a recombinant antibody by transient expression in plant leaves, stable transgenic plants are generated for long-term production of a required antibody. The stable transformation efficiency of plants depends on the particular plant species and variety, and it can take 3–9 months for plants to be ready for the evaluation of expressed protein.

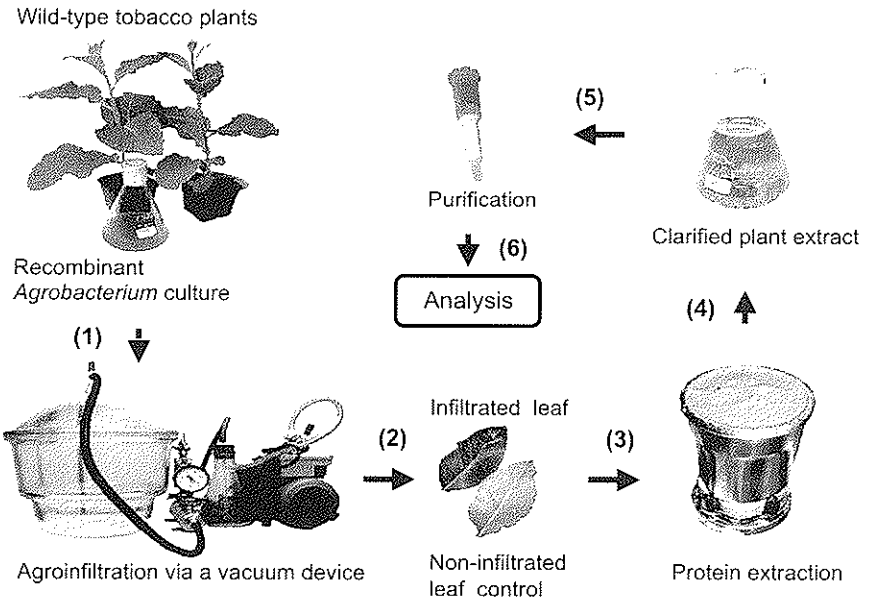

Figure 13.3. Transient assay in plants by agroinfiltration. Tobacco plants of wild type cultivated in greenhouse and recombinant *Agrobacterium* cell cultures transformed with a vector containing antibody gene of interest are prepared for transient assay. (1) Tobacco leaves are immersed in *Agrobacterium* cell suspensions and infiltration is carried out by a continuous vacuum in the range of 60–80 mbar for 15–20 min. (2) The infiltrated leaves are washed with tap water and incubated at 22°C under light for three days. Non-infiltrated leaves are used as controls for comparison. (3) Leaves are subjected to protein extraction in appropriate buffer. (4) The extracts are clarified by centrifugation. (5) Target antibodies from the supernatants are purified by affinity-chromatography. (6) The purified antibodies are used for subsequent analyses, such as specificity and affinity assays.

Transient expression is a prerequisite to stable transformation since it tests both expression vectors and protein stability and could identify potential problems that can be eliminated, so that the likelihood of generating the desired transgenic plants is significantly improved.

Since the time that functional full-size antibodies were first stably expressed in transgenic plants in 1989 (Hiatt *et al.*, 1989) and 1990 (Duering *et al.*, 1990), different forms of full-size IgG (Drake *et al.*, 2003) and secretory IgA (Nicholson *et al.*, 2005) antibodies, Fab fragments (de Neve *et al.*, 1993), scFv antibodies (Boonrod *et al.*, 2004), biscFv antibodies (Fischer *et al.*, 1999c), diabodies (Kathuria *et al.*, 2002), and scFv antibody fusions to AFPs (Peschen *et al.*, 2004) have been expressed in leaves and seeds of plants without loss of binding specificity or affinity (*Figure 13.4*). Exploiting the innate protein sorting and targeting mechanisms that plant cells normally use for targeting can enhance expression levels of recombinant antibodies in plants. Significant increases in recombinant antibody yield have been observed when antibodies are targeted to the secretory pathway instead of the cytosol (Conrad and Fiedler, 1998). Secretion of antibodies to the intercellular space leads to significant levels of expression, and ER retention can give 10- to 100-fold higher yields. Most plant-produced recombinant antibodies have been targeted to the following compartments of plant cells: the intercellular space, chloroplasts, and

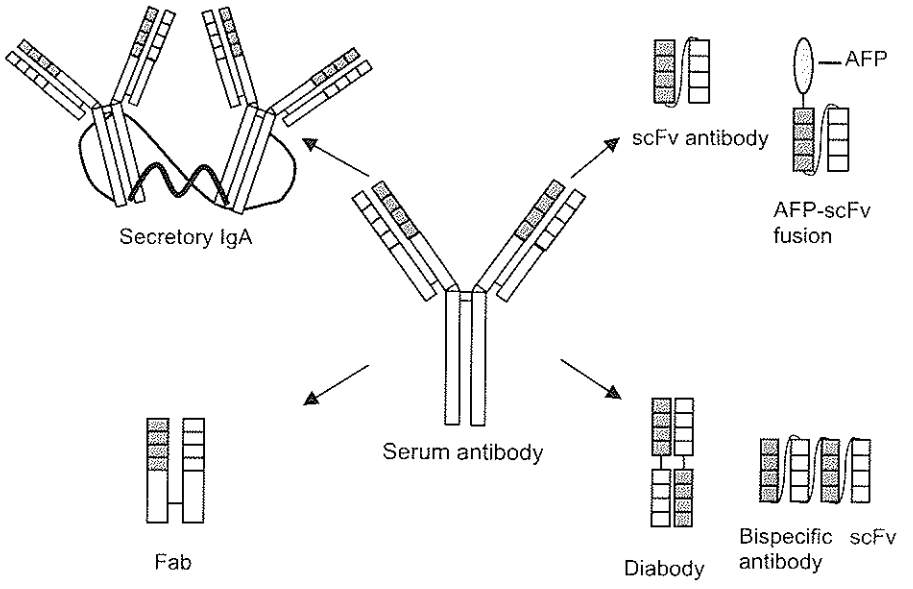

Figure 13.4. Different antibody formats expressed in transgenic plants.

endoplasmic reticulum (Duering *et al.*, 1990; Spiegel *et al.*, 1999; Boonrod *et al.*, 2004). Intracellular expression of recombinant antibodies in the cytoplasm has been achieved using only scFv fragments (Tavladoraki *et al.*, 1993; Zimmermann *et al.*, 1998), presumably because scFv fragments require only minor post-translational processing. In most cytosolic expressions of scFvs, levels were found to be very low or just at the detection limit (Xiao *et al.*, 2000; Santos *et al.*, 2004). A notable exception is an scFv antibody isolated by phage display that accumulated up to 1.0% of TSP in the plant cytosol (de Jaeger *et al.*, 1998). Given the fact that the expression level of some proteins reaches more than 46% of TSP in leaf tissues (Cosa *et al.*, 2001), antibody expression levels in plants may be increased further in the future by the use of stronger tissue or inducible promoters, improvement of transcript stability, translational enhancement with novel sequences or strategies, and transgenic chloroplast system.

In addition to the subcellular compartments, expression levels in stably transformed plants also vary depending on the antibodies and plant tissues, with expression of full-size IgG ranging from 0.35% (van Engelen *et al.*, 1994) to 1.1% of TSP in tobacco leaves (Vine *et al.*, 2001). An even higher level of expressed scFvs has been reported in transgenic plants reaching up to 500 μg secretory IgA per gram leaf tissues (Ma *et al.*, 1995) and up to 4.0% scFv antibody of the TSP in seeds (Phillips *et al.*, 1997). It has been shown that leaves from transgenic plants expressing ER-retained scFvs can be dried and stored for more than three weeks without loss of scFv specificity or antigen-binding activity (Fiedler *et al.*, 1997), and cereal seeds expressing antibodies can be stored at room temperature for two years without significant loss of functional antibody content (Stoeger *et al.*, 2002).

Plant-produced antibodies for human immunotherapy have already reached clinical trials (Ma *et al.*, 2005; Weintraub *et al.*, 2005), and plantibodies specific for

plant pathogens have been shown to provide effective control of diseases caused by the pathogens, as discussed in more detail below.

Antibody-mediated pathogen resistance in plants

Plant-produced antibodies have been developed, in many cases, for therapeutic applications, but they have also been used for immunomodulation (Miroshnichenko *et al.*, 2005) and the protection of plants against different pathogens including viruses, bacteria, and fungi (*Table 13.1*). For example, cytoplasmic expression of an scFv antibody against AMCV in transgenic tobacco has been shown to reduce viral infection and delay the progression of disease symptoms (Tavladoraki *et al.*, 1993). Also, secretion into the apoplast of a full-size antibody recognizing intact tobacco mosaic virus particles has been shown to reduce the number of local necrotic lesions in transgenic tobacco (Voss *et al.*, 1995). Another example is the expression of an scFv antibody specific for the stolbur phytoplasma major membrane protein, which enhanced plant resistance to phytoplasma pathogens (le Gall *et al.*, 1998). A more recent development has demonstrated antibody-mediated resistance against fungal pathogens in transgenic plants (Peschen *et al.*, 2004). The plantibody approach has several advantages over pathogen-derived resistance in transgenic plants that may create more virulent pathogens via genetic recombination (Aaziz and Tepfer, 1999).

USING RECOMBINANT ANTIBODIES TO PROTECT PLANTS FROM VIRAL PATHOGENS

There are about 500 known viruses that attack and cause diseases in plants. With a small genome and a simple infection cycle, a plant virus multiplies only in living cells, and the viral-encoded proteins responsible for infection are known. Thus, they appear to be ideal pathogens for generating resistant plants using the plantibody approach. Three basic viral antigenic targets for antibodies could be: (1) coat protein; (2) replicase or polymerase and protease domains; and/or (3) movement proteins. Neutralization or disruption of any of these proteins, in principle, should generate virus-resistant plants. The feasibility of recombinant antibodies to interfere in the infection of a plant virus was demonstrated in 1993 (Tavladoraki *et al.*, 1993). In this case, the expression of a cytosolic scFv fragment against the coat protein of AMCV caused a reduction in viral infection and a delay in the development of symptoms. Since most processes involved in viral infection, replication, and dissemination take place within the plant cytosol (Baulcombe, 1994), cytosolic expression of the recombinant antibodies has been favoured. A disadvantage of the cytosolic antibody approach is that scFvs have low stability in the plant cytosol. Despite this, remarkably enhanced local and systemic viral resistance has been reported for transgenic tobacco plants expressing a very low level of cytosolic scFv (0.00002% of total soluble protein) specific to TMV (Zimmermann *et al.*, 1998). In contrast, targeting of TMV-specific scFv antibodies or full-size antibodies to the apoplast gave a lower degree of resistance, although the apoplast-targeted, full-size antibodies accumulated to a 50 000-fold higher level than a cytosolic scFv (Zimmermann *et al.*, 1998). These results demonstrate that selection of the plant compartment for antibody expression is important for engineering viral resistance.

Nevertheless, viruses initially infect plant cells through the intercellular space,

Table 13.1. Plantibody-mediated resistance to pathogens in transgenic plants

Pathogen/antigen	Antibody	Location	Target plant	Biological effect	Reference
Artichoke mottled crinkle virus/coat protein	scFv	cytosol	*Nicotiana tabacum*	reduction of infection; delay in symptom development	Tavladoraki *et al.*, 1993
Botrytis cinerea/cutinase	full-size	apoplast	*N. tabacum*	not reported	van Engelen *et al.*, 1994
Tobacco mosaic virus/coat protein	full-size	apoplast	*N. tabacum* cv. Xanthi nc	70% reduction of local necrotic lesion number	Voss *et al.*, 1995
Root-knot nematode/stylet secretions	full-size	apoplast	*N. tabacum* cv. Xanthi	no effect on nematode infection – probably due to mistargeting of the antibody	Baum *et al.*, 1996
Beet necrotic yellow vein	scFv	ER	*N. benthamiana*	delay in symptom development	Fecker *et al.*,1997
Tobacco mosaic virus/coat protein	scFv	cytosol	*N. tabacum* cv. Xanthi nc	92% reduction of local necrotic lesion number in viral infection, 11% of transgenic plants were fully resistant in systemic infection assays	Zimmermann *et al.*, 1998
Stolbur phytoplasma/major membrane protein	scFv	apoplast	*N. tabacum*	free of symptoms	le Gall *et al.*, 1998
Corn stunt spiroplasma/ membrane protein	scFv	cytosol	*Zea mays*	no resistance	Chen and Chen, 1998
Tomato spotted wilt tospovirus/ glycoprotein G1	scFv	apoplast	*N. benthamiana*	fully functional for specificity and affinity, and resistance assay is not reported	Franconi *et al.*, 1999
Potato virus strain Y and D, clover yellow vein virus strain 300/coat protein	scFv	cytosol, apoplast	*N. tabacum* cv. W38	reduction of local necrotic lesions and systematic symptoms	Xiao *et al.*, 2000
Tobacco mosaic virus/coat protein	scFv	plasmalemma membrane surface	*N. tabacum* cv. Petite Havana SR1	16% of transgenic plants were fully resistant in systemic infection assays	Schillberg*et al.*, 2000
Fusarium/cell wall protein	scFv, AFP–scFv fusion	apoplast	*Arabidopsis thaliana*	78% reduction of infection 21 days post inoculation with *Fusarium oxysporum*	Peschen *et al.*, 2004
Tomato bushy stunt virus/RNA dependent RNA polymerase	scFv	cytosol,ER	*N. benthamiana*	complete resistance to TBSV and CNV; partial resistance to TCV and RCNMV	Boonrod *et al.*, 2004
Stolbur phytoplasma/main membrane protein	scFv	apoplast	*N. tabacum*	delay in symptom appearance	Malembic-Maher *et al.*, 2005

CNV, cucumber necrosis virus; RCNMV, red clover necrotic mosaic virus; TBSV, tomato bushy stunt virus; TCV, turnip crinkle virus.

and hence interfering in viral entry may affect virus infectivity at the first barrier in plant cells. Based on that assumption, plant-produced antibodies are also targeted to the apoplast. Indeed, secretion of virus-specific antibodies into the apoplast has led to an enhanced resistance in several instances, presumably by directly interfering with viral entry (Voss *et al.*, 1995; Fecker *et al.*, 1997; Xiao *et al.*, 2000). Another example is to target anti-TMV antibodies to the plasma membrane *in planta*, resulting in transgenic plants resistant to TMV infection (Schillberg *et al.*, 2000). The membrane-anchored antibodies may efficiently block viral infection because plasma membrane is the location where the entrance of the virions into the cell normally takes place. Therefore, plant cell surface expression in combination with cytosolic expression of virus-specific antibodies may reduce viral infection and spreading with more effectiveness and durability.

Selection of viral coat proteins as antigens for the generation of recombinant antibodies might have the potential for drawbacks. Plant viral coat proteins have a broad structural diversity and this restricts the effect of the expressed antibodies to a small range of viruses. In addition, the viral coat protein sequence can alter under selective pressure to overcome coat protein-based resistances. Therefore, generation of recombinant antibodies directed against conserved functional domains of viral replicases or polymerases and transport proteins could provide a better route for engineering pathogen-resistant plants possessing a broad spectrum and durable resistance against viruses. Boonrod and colleagues have demonstrated this recently (Boonrod *et al.*, 2004). These researchers demonstrated that transgenic tobacco plants expressing scFv antibodies against a conserved domain in a plant viral RNA-dependent RNA polymerase, either in the cytosol or in the ER, showed high levels of resistance to four plant viruses from different genera. These results have paved the way for engineering broad-range virus resistance by the expression *in planta* of scFv antibodies specific for highly conserved motifs in viral replicases or polymerases. Another strategy exploiting bivalent biscFv antibodies (Fischer *et al.*, 1999c), with two different specificities – one viral capsid specific and a second specific for either a replicase or movement protein – may generate improved resistance against plant viruses.

These recombinant antibody approaches can be combined to create plants with resistance being pyramided. With any one of the plantibody approaches described here, there are no potential risks for the creation of new virulent viruses that other pathogen-derived resistance strategies may create (Aaziz and Tepfer, 1999).

USING RECOMBINANT ANTIBODIES TO PROTECT PLANTS FROM MOLLICUTE PATHOGENS

Mollicutes (phytoplasmas and spiroplasmas) are wall-less, phytopathogenic bacteria that cause more than 300 diseases of plants. They have originated from low-G+C Gram-positive bacteria by gene loss and genome reduction, and have lost the genes responsible for synthesis of a bacterial cell wall. Thus, only a single cytoplasmic membrane delimits phytoplasmas, and this has led to growth inhibition of the pathogens by antibodies directed against their membrane epitopes (Malembic *et al.*, 2002). Therefore, mollicutes are considered to be an ideal target for the plantibody-mediated resistance approach.

Expression of scFv antibodies specific for the major membrane protein of the stolbur phytoplasma in transgenic tobacco plants confers a protection of plants from the pathogen (le Gall *et al.*, 1998). In this case, the scFv construct carries a bacterial leader peptide, *pelB*, and the expressed scFvs in transgenic plants seem to enter the secretory pathway, since in the transgenic plant phloem tissue, phytoplasma-specific scFv antibodies were indeed shown to be present, as revealed by immunofluorescence detection. Phytoplasmas are located in plant phloem tissues. The transgenic tobacco shoots grafted on phytoplasma-infected rootstocks grew free of symptoms, while control plants showed severe disease symptoms (le Gall *et al.*, 1998). A recent report has shown that the apoplast-targeted scFvs specific for phytoplasma pathogen were functional and the expression level reached up to 0.04% of TSP. The transgenic plants expressing secreted scFvs displayed a reduction of susceptibility to the pathogen (Malembic-Maher *et al.*, 2005). Efficiently targeting plantibodies or heterologous proteins to plant phloem in transgenic plants has been a major challenge (Foissac *et al.*, 2000), and further investigations on new targeting sequences and functional characterization of targeting different plantibodies to phloem sap will promote applications of plantibodies in mollicute resistance.

USING RECOMBINANT ANTIBODIES TO PROTECT PLANTS FROM FUNGAL PATHOGENS

Pathogenic fungi are eukaryotic microorganisms that have acquired the capacity to grow on living plants. More than 10 000 species of fungi can cause diseases in plants, and all plants are attacked by some fungi. Fungal pathogens have developed machinery to circumvent a multitude of defence mechanisms of the host plants. Fungi infecting plants procure their nutrients from the host plants and spread mycelia into plant tissues. During the infection process, fungi have an intimate interaction with plant cells and secrete various proteins essential for fungal pathogenesis. Blockage of the function of these compounds or fungal cell components with antibodies may affect fungal pathogen development and dissemination. This will reduce the damage caused by fungi without the environmental pollution that occurs when fungicides are used to control fungal diseases. Antibodies have been generated against fungal conidiospores, secreted proteins or compounds, cell wall fragments, cell surface antigens of mycelia, and infection structures *in planta* of fungi (Robert *et al.*, 1993; Goebel *et al.*, 1995; Murdoch *et al.*, 1998; Peschen *et al.*, 2004; Manatunga *et al.*, 2005).

Application of antibodies to prevent fungal infection was first reported by Maiti and Kolattukudy (1979). Polyclonal antibodies prepared against a cutinase from *Fusarium solani pisi* effectively prevent infection of the host by this fungus. Similarly, it has been shown that *Colletotrichum gloeosporioide* symptom development in avocado, mango, and banana can be inhibited by the use of polyclonal antibodies directed against the fungal pectate lyase (Wattad *et al.*, 1997). Monoclonal antibodies specific for a cell wall-bound component of *Neotyphodium coenophialum* effectively inhibits the fungal growth in cultures (Hiatt *et al.*, 2001). scFv antibodies directed against extracellular proteins from *Rhizoctonia solani* isolated from a phage display library inhibited polygalacturonase in the culture supernatants of a

range of fungal pathogens, including ascomycetes, basidiomycetes, and oomycetes. The phage-derived scFv antibodies also inhibited maceration of potato tissue by these pathogens (Manatunga *et al.*, 2005).

To genetically engineer plant resistance against fungal pathogens, several genes capable of inhibiting fungal growth have been expressed in plants. Most efforts have been directed toward expression of AFPs such as chitinases (Punja, 2001). This procedure has shown the ability of AFPs to provide plant resistance to fungal pathogens. However, resistance by the expression of AFPs is not specific to any one fungal species. Moreover, expression of AFPs in transgenic plants only delays the appearance of disease symptoms and does not provide effective control of the disease. Therefore, development of novel, specific, targeted molecules to control fungal pathogens opens a promising avenue for the improvement of plant fungal resistance. This approach is particularly significant for many agriculturally important fungi, such as *Fusarium* species infecting cereal crops, for which there are no highly resistant varieties available. Currently, these fungal diseases are controlled mainly by the application of chemical fungicides (Windels, 2000).

Recently, we have demonstrated for the first time antibody-mediated fungal resistance in transgenic plants by generating specific antibodies against *Fusarium graminearum*, a predominant fungal species infecting wheat and other cereals in China and other countries (Wu *et al.*, 2005). Natural resistance against this mycotoxin-producing fungus is inadequate, and no germ plasm exists that provides effective innate resistance to this pathogen under high disease pressure. Our strategy is based on the use of chicken antibodies that specifically target AFPs to the site of infection by binding to surface components of the invading fungus, thus interfering with fungal growth and development. To generate antibodies specific for *F. graminearum*, fungal cell wall proteins and germinated spores were prepared from the fungus and used to immunize chickens. *Fusarium*-specific scFv antibodies were isolated by phage display following the establishment of a pooled immunocompetent library. The scFv antibodies generated revealed a high specificity and affinity toward *Fusarium* fungi and bound to one protein with a molecular mass of approximately 50 kDa from cell wall preparations of *F. graminearum* and *F. oxysporum* f.sp. *matthiolae*. Confocal immunofluorescence labelling provided further evidence that those scFv antibodies bound specifically to a cell wall surface target. After transient assay by agroinfiltration, one scFv antibody, CWP2, was selected and stably expressed in transgenic *Arabidopsis* plants. The results indicated that expression of the CWP2 scFv antibody conferred significant resistance *in planta* upon the challenge by *F. oxysporum* f.sp. *matthiolae*, and similar to that of expressing AFPs under the same conditions (Peschen *et al.*, 2004).

More importantly, when the coding sequence of CWP2 antibody was genetically fused to any of three AFPs, the resulting AFP–scFv fusion proteins (*Figure 13.4*) showed strong inhibitory effects on the growth of *Fusarium* spp. *in vitro*, and transgenic *Arabidopsis thaliana* plants expressing the fusions showed very significantly enhanced resistance to the pathogens. The three AFPs used are a chitinase from wheat, an anti-fungal peptide from *Raphanus sativus* capable of interfering with membrane potential by interacting with the plasma membrane proton pump, and a positively charged peptide from *Aspergillus giganteus*, which is thought to interact with negatively charged phospholipids of fungal membranes. Despite the

integration of the AFPs into the fusions, this antibody-mediated resistance is *Fusarium*-specific because the fusion proteins do not provide resistance to a non-*Fusarium* fungus, *Sclerotinia sclerotiorum* (Peschen *et al.*, 2004). These results clearly validate that the *Fusarium*-specific scFv antibody CWP2 was essential for enhanced *Fusarium*-specific resistance *in planta*.

As to the action mode or mechanism displayed by the scFv–AFP fusions in the fungal resistance process, we assume that the AFP–scFv fusion proteins generated combine the specificity and affinity of an scFv antibody with the anti-fungal activity from an AFP, and thus could accumulate to a high concentration at their normal site of action due to CWP2 scFv antibody binding to surface antigens of the invading fungus in plants. Under such a situation, AFPs could directly degrade the fungal cell wall (e.g. chitinase) or interfere with membrane potential, and at the same time, CWP2 could also interfere with the fungal development by blocking the fungal antigen to which the antibody binds (Bohlmann, 2004). Our results have demonstrated that engineered antibody-fusion proteins expressed in transgenic plants can control *Fusarium* infection, providing new opportunities for the development of environment-friendly pathogen resistance management practices. Further investigations with transgenic *Arabidopsis* and wheat are under way in our laboratory with the aim of revealing *in planta* binding targets for the fungal antibody and understanding the biological roles played by the cell surface antigen of *Fusarium* fungi, which interacts with the antibody during pathogenesis.

Conclusion

Plants have been utilized for the expression of antibodies specific for pathogenic viruses, mollicutes, nematodes, and fungi. The expressed plantibodies retain their functionality and properties as their hybridoma-derived counterparts, and convey endogenous resistance for plants. This is particularly important for breeding cultivars resistant to pathogens, for which no innate resistance germ plasm is available in nature. Successful exploitation of plantibodies to create pathogen-resistant plants depends on appropriate antigen selection, antibody design, antibody selection, antibody stability and antibody expression, and targeting to appropriate cellular compartments. Development of phage display and ribosome display has allowed the generation of recombinant antibodies directed against any targets within a few weeks in conventional molecular biology laboratories. A high level of expression and specific targeting of pathogen-specific antibodies and thereof in transgenic plants will further increase the efficacy of plantibodies. Technical advances of these approaches certainly will bring more applications into plant science and biotechnology. We envisage that plant-produced antibodies will become one of the predominant strategies for the protection of crop plants against pathogens in the coming decade.

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