Plant diseases have a significant impact on the yield and quality of crops. Many strategies have been developed to combat plant diseases, including the transfer of resistance genes to crops by conventional breeding. However, resistance genes can only be introgressed from sexually-compatible species, so breeders need alternative measures to introduce resistance traits from more distant sources. In this context, genetic engineering provides an opportunity to exploit diverse and novel forms of resistance, e.g. the use of recombinant antibodies targeting plant pathogens. Native antibodies, as a part of the vertebrate adaptive immune system, can bind to foreign antigens and eliminate them from the body. The ectopic expression of antibodies in plants can also interfere with pathogen activity to confer disease resistance. With sufficient knowledge of the pathogen life cycle, it is possible to counter any disease by designing expression constructs so that pathogen-specific antibodies accumulate at high levels in appropriate sub-cellular compartments. Although first developed to tackle plant viruses and still used predominantly for this purpose, antibodies have been targeted against a diverse range of pathogens as well as proteins involved in plant–pathogen interactions. Here we comprehensively review the development and implementation of antibody-mediated disease resistance in plants.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction
Food security is one of the major socioeconomic challenges we face in the 21st century as the human population grows while farmland and water reserves diminish. Although technological developments in agriculture have increased yields significantly over the last few decades, even more productivity is required to meet the growing demand for food now and in the future (James, 2009). One of the greatest constraints affecting agricultural productivity is the impact of plant diseases, caused by viruses, bacteria, fungi and nematodes. In the worst scenarios, diseases can destroy crops completely, with a devastating impact on farmers and consumers. On average, plant diseases are responsible for 14.1% yield losses in the agricultural sector, costing approximately US$220 billion per year (Agrios, 2005). In developing countries, the percentage losses
are considerably higher because only limited technology and infrastructure are available for disease prevention and monitoring.

Strategies to attenuate or control plant pathogens often rely on the development of resistant cultivars by conventional breeding, the chemical control of pathogens and their vectors, or the adoption of preventative agricultural practices. Genetic engineering and biotechnology now offer additional opportunities for disease prevention because they allow disease-resistance genes to be introduced into plants from diverse sources, with none of the species barriers that apply to conventional strategies. For example, the expression of viral nucleic acid sequences in plants can often induce resistance against the same virus and its relatives (pathogen-mediated resistance). Such sequences can include the coat protein, movement protein or replicase protein genes, but also non-coding sequences (Prins et al., 2008; Ritzenhailer, 2005; Sudarshana et al., 2007; Vanderschuren et al., 2007). The use of pathogen genes as a defense strategy is not always suitable because this approach does not work against DNA viruses, and there is also the risk that new pathogen strains could arise by genetic recombination or transcapsidation if longer transgenes are used (the risk is very low with shorter sequences, such as minigenes producing hairpin RNAs). Alternative approaches include the expression of non-pathogen proteins with anti-microbial activity, such as ribonucleases (Trifonova et al., 2007; Watanabe et al., 1995), 2′,5′-oligoadenylate synthases (Truve et al., 1993) and ribosome-inactivating proteins (Nielsen and Boston, 2001; Wang and Tuner, 2000) for the inhibition of viruses, and proteins related to osmotin (Ibeas et al., 2001; Monteiro et al., 2003; Zhu et al., 1996) and thraumatin (Anand et al., 2003; Chen et al., 1999; Monteiro et al., 2003) for the inhibition of fungi.

The ectopic expression of antibodies in plants is an extension of the above strategy, but antibodies can be designed to target any pathogen (Table 1). Full-size antibodies and their fragments produced in planta above strategy, but antibodies can be designed to target any pathogen (Monteiro et al., 2003; Zhu et al., 1996) and thaumatin (Anand et al., 2007; Vanderschuren et al., 2007). The use of pathogen genes as a defense strategy is not always suitable because this approach does not work against DNA viruses, and there is also the risk that new pathogen strains could arise by genetic recombination or transcapsidation if longer transgenes are used (the risk is very low with shorter sequences, such as minigenes producing hairpin RNAs). Alternative approaches include the expression of non-pathogen proteins with anti-microbial activity, such as ribonucleases (Trifonova et al., 2007; Watanabe et al., 1995), 2′,5′-oligoadenylate synthases (Truve et al., 1993) and ribosome-inactivating proteins (Nielsen and Boston, 2001; Wang and Tuner, 2000) for the inhibition of viruses, and proteins related to osmotin (Ibeas et al., 2001; Monteiro et al., 2003; Zhu et al., 1996) and thraumatin (Anand et al., 2003; Chen et al., 1999; Monteiro et al., 2003) for the inhibition of fungi.

Table 1
Recombinant antibody-mediated resistance against plant diseases.

<table>
<thead>
<tr>
<th>Year</th>
<th>Disease agent</th>
<th>Targeted protein</th>
<th>Transformed species</th>
<th>rAb format</th>
<th>Cellular localization</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1993</td>
<td>ACMV</td>
<td>Coat protein</td>
<td>Nicotiana benthamiana</td>
<td>scFv</td>
<td>Cytosol</td>
<td>Tavladoraki et al., 1993</td>
</tr>
<tr>
<td>1995</td>
<td>TMV</td>
<td>Coat protein</td>
<td>N. tabacum cv. Xanthi</td>
<td>full size IgG</td>
<td>Apoplast</td>
<td>Voss et al., 1995</td>
</tr>
<tr>
<td>1997</td>
<td>BNYVV</td>
<td>Coat protein</td>
<td>N. benthamiana</td>
<td>scFv</td>
<td>ER</td>
<td>Fecker et al., 1997</td>
</tr>
<tr>
<td>1998</td>
<td>StaLb phytplasma</td>
<td>IMP</td>
<td>N. tabacum</td>
<td>scFv</td>
<td>Cytosol</td>
<td>Le Gall et al., 1998</td>
</tr>
<tr>
<td>2000</td>
<td>PVY strains Y&amp;D</td>
<td>Coat protein</td>
<td>N. tabacum cv. W38</td>
<td>scFv</td>
<td>Apoplast, cytosol</td>
<td>Xiao et al., 2000</td>
</tr>
<tr>
<td>2001</td>
<td>TMV</td>
<td>Coat protein</td>
<td>N. tabacum cv. Samsun NN</td>
<td>scFv</td>
<td>Cytosol</td>
<td>Bajorovic et al., 2001</td>
</tr>
<tr>
<td>2004</td>
<td>Fusarium oxysporum f. sp. matthiolae</td>
<td>Cell-wall bound proteins</td>
<td>Arabidopsis thaliana</td>
<td>scFv-APF</td>
<td>Apoplast</td>
<td>Peschen et al., 2004</td>
</tr>
<tr>
<td>2004</td>
<td>TBSV, CNV, TCV, RCNMV</td>
<td>RdRp</td>
<td>N. benthamiana</td>
<td>scFv</td>
<td>Cytosol, ER</td>
<td>Boonrod et al., 2004</td>
</tr>
<tr>
<td>2005</td>
<td>StaLb phytplasma</td>
<td>IMP</td>
<td>N. tabacum</td>
<td>scFv</td>
<td>Apoplast, cytosol</td>
<td>Malenica-Maher et al., 2005</td>
</tr>
<tr>
<td>2005</td>
<td>CMV</td>
<td>Coat protein</td>
<td>N. benthamiana</td>
<td>scFv</td>
<td>Cytosol</td>
<td>Villani et al., 2005</td>
</tr>
<tr>
<td>2005</td>
<td>TSWV</td>
<td>Nucleoprotein</td>
<td>N. benthamiana</td>
<td>scFv</td>
<td>Cytosol</td>
<td>Prins et al., 2005</td>
</tr>
<tr>
<td>2006</td>
<td>PVY</td>
<td>Nla protein</td>
<td>Solanum tuberosum</td>
<td>scFv</td>
<td>Cytosol</td>
<td>Gargouri-Bouzid et al., 2006</td>
</tr>
<tr>
<td>2008</td>
<td>PLRV</td>
<td>P1 protein</td>
<td>S. tuberosum</td>
<td>scFv</td>
<td>Cytosol</td>
<td>Nickel et al., 2008</td>
</tr>
<tr>
<td>2008</td>
<td>TSWV</td>
<td>Movement protein</td>
<td>N. tabacum cv. Petit Havana SR1</td>
<td>scFv</td>
<td>Cytosol</td>
<td>Zhang et al., 2008</td>
</tr>
<tr>
<td>2008</td>
<td>F. asiaticum</td>
<td>Cell-wall bound proteins</td>
<td>Triticum aestivum</td>
<td>scFv-APF</td>
<td>Apoplast</td>
<td>Li et al., 2008</td>
</tr>
<tr>
<td>2009</td>
<td>GFLV, AvMV</td>
<td>Coat protein</td>
<td>N. benthamiana</td>
<td>scFv</td>
<td>Cytosol</td>
<td>Nölke et al., 2009</td>
</tr>
<tr>
<td>2009</td>
<td>PVY</td>
<td>Nla protein</td>
<td>S. tuberosum</td>
<td>scFv</td>
<td>VH</td>
<td>Bouaziz et al., 2009</td>
</tr>
<tr>
<td>2009</td>
<td>TYLCV</td>
<td>Rep</td>
<td>N. benthamiana</td>
<td>scFv-GFP</td>
<td>Cytosol</td>
<td>Safarnejad et al., 2009</td>
</tr>
<tr>
<td>2010</td>
<td>CTV</td>
<td>p25 major coat protein</td>
<td>Citrus aurantifolia</td>
<td>scFv</td>
<td>Cytosol</td>
<td>Cervera et al., 2010</td>
</tr>
<tr>
<td>2010</td>
<td>Sclerotinia sclerotiorum</td>
<td>Highpro proteins</td>
<td>Brassica napus</td>
<td>scFv</td>
<td>Cytosol</td>
<td>Yajima et al., 2010</td>
</tr>
<tr>
<td>2011</td>
<td>PPV</td>
<td>Nib protein</td>
<td>N. benthamiana</td>
<td>scFv</td>
<td>Cytosol, ER, nucleus</td>
<td>Gil et al., 2011</td>
</tr>
</tbody>
</table>

In this article, we discuss methods used to create and express pathogen-specific antibodies and experiments that have established and developed the principle of antibody-mediated disease resistance in plants.

2. Antibody structure

Antibodies exist both as soluble proteins and as membrane-bound receptors on the surface of B-lymphocytes. Their primary function is antigen binding, and this often has a direct inhibitory effect on the pathogen target, e.g. the neutralization of a bacterial toxin or the prevention of viral attachment to host cells. Such effects are independent of the immunoglobulin isotype because only the variable regions of the antibody contact the antigen, hence they function equally well in heterologous settings such as plants. In mammals, antibodies also elicit additional responses through the effector functions conferred by the constant regions. These are dependent on the immunoglobulin isotype and they activate immunological cascades such as the classical pathway of the complement system. Effector functions are irrelevant for applications in plants because the corresponding pathways are absent, so we do not discuss them further.

All immunoglobulins have a similar structure, consisting of two identical heavy chains (HCs) and two identical light chains (LCs). The HC comprises 440–450 amino acid residues (MW = 50 kDa) whereas the smaller LC comprises 220 residues (MW = 25 kDa). Each HC is attached to a LC via disulfide bonds, with additional disulfide bonds in the hinge region joining the two HCs together to complete the tetrameric structure (Fig. 1). Further intramolecular disulfide bonds help the antibody to fold correctly into globular domains each containing 110 residues. The C-terminal portions of the HC and LC show limited variability and are known as constant regions. The LC has a single constant region (C\text{\text{L}}) whereas the HC has three (C\text{\text{H}}\text{\text{1}}, C\text{\text{H}}\text{\text{2}} and C\text{\text{H}}\text{\text{3}}), each forming an independent structural domain. In contrast, the N-terminus 110 residues of both the HC and LC are variable regions (V\text{\text{H}} and V\text{\text{L}}) that confer antigen-binding specificity. Hypervariable regions, also known as complementary determining regions (CDRs), are found within V\text{\text{H}} and V\text{\text{L}}, and these make direct contact with the antigen (Kabat et al., 1977). Outside the CDRs are more conserved framework
regions that maintain the integrity of antibody–antigen binding (Morea et al., 1997, 2000).

Antibody–antigen interactions involve multiple non-covalent bonds, the strength of which depends on the distance between interacting chemical groups. In some cases, several non-covalent bonds can form simultaneously and the opposing interacting units (the epitope on the antigen and its cognate paratope on the antibody) are complementary and have sufficient binding energy to resist thermodynamic disruption. The attraction between a single epitope and its corresponding paratope is known as antibody affinity, whereas the combined strength with which a multivalent antibody binds a multivalent antigen is the antibody avidity. Antibodies recognize the overall shape of an epitope rather than particular residues, and can distinguish minor differences in the primary amino acid sequence of antigens as well as differences in charge, optical configuration and steric conformation. Many antibodies will bind only to native antigens or fragments that have folded properly to form multiple non-linear interactions between the antigen and antibody. Antibodies that bind to such discontinuous epitopes often do not bind denatured antigens (Sakurabayashi, 1995).

Antibodies are remarkably diverse and have the capacity to recognize tens of millions of different antigens in the environment. This diversity is achieved by somatic gene rearrangement, reflecting the fact that germ line immunoglobulin genes exist as multiple segments that are combined in different ways in different B-cells. The germ line LC gene comprises up to 200 alternative V (variable) gene segments separated from the C (constant) region by five alternative J (joining) segments. During B cell differentiation, one of the V gene segments joins one of the J gene segments to form the VJ region (Melchers, 1995). The germ line HC gene is even more complex, comprising three groups of gene segments upstream of the constant region (V, D and J; D for diversity). There are 200 V gene segments, 12 D gene segments and four J gene segments, and during B-cell differentiation one of each segment is joined to the C region to form the mature HC gene (Early et al., 1980). Through these rearrangements, approximately $10^{4}$ different light chain genes and $10^{4}$ heavy chain genes can be formed, leading to the creation of $10^{7}$ different types of antibodies. Even more diversity can be introduced by variable splicing positions during recombination and the introduction of point mutations into the coding sequence by a process known as somatic hypermutation.

Partial digestion of a full-size immunoglobulin generates antibody fragments that remain biologically active. For example, digestion with papain creates one Fc and two Fab fragments, whereas digestion with pepsin creates a F(ab′)$_2$ fragment containing two Fab sequences and the hinge region (Fig. 1). Full or partial antibody genes can be cloned and expressed in heterologous systems, allowing the above fragments (and others) to be expressed as truncated polypeptides (Zimmermann et al., 2006). Full-size antibodies are usually multivalent, enabling them to bind to their targets with a high avidity, whereas fragments may be multivalent or univalent depending on their construction. Monovalent single-chain variable fragments (scFvs) comprise the variable regions of the HC and LC joined by a short linker peptide that is usually rich in glycine and serine (Fig. 1). These have the least stringent requirements for folding and assembly, and also penetrate tissues effectively because of their small size. This makes them suitable for expression in diverse environments, such as the various intracellular compartments in plant cells. Bispecific single-chain fragments comprise two individual scFvs connected to each other by another peptide linker (Fig. 1). Furthermore, multifunctional recombinant antibody fragments can be generated by coupling an antibody gene fragment to a gene encoding an unrelated protein domain such as a toxin, biological response modifier or enzyme (Fan et al., 2002; Niv et al., 2003; Li et al., 1999; Peschen et al., 2004; Rau et al., 2002).

3. Generating pathogen-specific antibodies

Hybridoma technology is the traditional way to generate valuable monoclonal antibodies, and this involves the immortalization of antigen-specific B-lymphocytes (Köhler and Milstein, 1975). The advent of hybridoma technology led to the development of research-grade antibodies that have accelerated the pace of basic research, and antibody-based drugs that have achieved considerable advances in medicine. The first antibodies against plant pathogens were generated using hybridoma technology, and have been expressed either as full-
size immunoglobulins or as antibody fragments in plants (Chen and Chen, 1998; Le Gall et al., 1998; Malemiche-Maher et al., 2005; Nölke et al., 2009; Orecchia et al., 2008; Schubert et al., 2011; Tavладоарки и др., 2003; Voss et al., 1995).

Despite these achievements, hybridoma technology is time-consuming and dependent on the availability of fully-equipped animal facilities. More recently, recombinant DNA and molecular display technologies have provided new opportunities for the creation of recombinant antibodies. Phage display, first described in 1985, involves the introduction of peptide sequences (such as the antigen-binding domains of recombinant antibodies) into the coat protein gene of a bacteriophage so that multiple copies of the peptide are displayed on the virion surface. Large peptide libraries, including diverse libraries of antibodies, can thus be prepared using phage display and screened by a process known as panning (Smith, 1985). Phage antibody display libraries can be screened with antigens to isolate antibodies that bind with the greatest affinity to the target, and the corresponding antibody gene can then be recovered from the phage genome for iterative rounds of modification and panning, resulting in antibodies that bind with exceptional affinity to the target antigen.

Most phage display platforms are based on filamentous bacteriophage such as M13, Fl and Fd, which lysogenize Escherichia coli and therefore form turbid rather than clear plaques on a bacterial lawn. They possess circular, covalently closed ssDNA genomes (approximately 6.4 kb in length) enclosed within a cylinder of coat proteins. Heterologous peptides are usually fused to the N-terminus of coat proteins pIII or pVIII, although the C-terminus of pV can also be used (Jesper et al., 1995). Each bacteriophage particle comprises up to 3000 copies of the pVIII major coat protein and 3–5 copies of the pIII minor coat protein, the latter required for infections because the C-terminal domain is necessary for the phage to escape from host cells (Rakonjac et al., 1999).

Phage display can be achieved using particles containing a whole phage genome plus the inserted peptide, or the phage particles may be used to transduce a phagemid (a plasmid carrying the phage origin of replication and one gene encoding a coat protein fusion). In the latter approach, superinfection with a helper phage is necessary to package the phagemid. This is preferable when the displayed protein is large enough to reduce the infectivity of the phage particles, to avoid packaging constraints. The minor coat protein pIII can tolerate N-terminal fusions with scFvs (McCafferty et al., 1990), but larger peptides such as Fab fragments are displayed more efficiently by fusion with the pVIII major coat protein (Kang et al., 1991).

Several scFv display libraries have been constructed in which the scFvs have been fused to the pIII minor coat protein. The genes encoding V_H and V_L can be amplified from antibody genes in donor species such as mice (Hoogenboom et al., 1991), humans (Sheets et al., 1998), rabbits (Ridder et al., 1995), cattle (O’Brien et al., 1999), sheep (Li et al., 2000), poultry (van Wyngaard et al., 2004) and camels (Hamers-Casterman et al., 1993) and then cloned in phagemid vectors. These genes can be obtained from the spleen or peripheral blood lymphocytes of immunized animals (Clackson et al., 1991) or the bone marrow, tonsils and peripheral blood lymphocytes of non-immunized donors (Sheets et al., 1998; Vaughan et al., 1996).

Camelid antibodies are particularly advantageous because of their unique structure, comprising two heavy chains, each consisting of a single monomeric variable domain (VHH), and no light chains (Muyldermans, 2001; Rahbarizadeh et al., 2011; Tillib, 2011). Camelid antibodies therefore have a much lower molecular weight than full-size immunoglobulins (12–15 kDa compared to 150 kDa), which has earned them the alternative name ‘nanobody’, yet they have similar affinity and binding specificity. They are even smaller than Fab fragments (50 kDa) and scFvs (25 kDa), but nevertheless display resistance to heat, detergents and concentrated urea because they do not require disulfide bonds for stability, making them suitable for cytosolic expression in plants. They are also highly soluble because a hydrophobic patch is covered by CDR3 (Rahbarizadeh et al., 2011).

Because V-gene repertoires for phage antibody display libraries can be obtained from immunized or non-immunized animals, two distinct library formats are available, described as ‘immunized’ and ‘naïve’ (Clackson et al., 1991; Marks et al., 1991). Immunized libraries are biased toward specific antibodies with high affinity for the target antigen, and they contain antibody genes representing the immune repertoire after somatic rearrangement of the germ line sequences (Burton et al., 1991; Clackson et al., 1991). Therefore, although relatively small libraries are sufficient to isolate high-affinity antibodies recognizing a given antigen, the major disadvantage is that a fresh library is required for each new target. In contrast, antibody sequences can be retrieved from off-the-shelf naïve libraries without prior immunization, sequence amplification and library construction. This allows the selection of antibodies against large panels of antigens, including those unsuitable for immunization, i.e. self-antigens, non-immunogenic antigens and toxic antigens (Griffiths et al., 1993; Marks et al., 1991; Okamoto et al., 2004; Park et al., 2005; Vaughan et al., 1996). Naïve libraries contain antibody sequences corresponding to the primary repertoire of the source organism, as well as memory B-cells unique to the individual source. The main disadvantage of naïve libraries is that much larger libraries are required to isolate high-affinity antibodies against particular antigens and the screening procedure is therefore more complex. Indeed, the affinity of the antibodies selected from a naïve library is proportional to the size of the library, ranging from 10^7 to 10^10 M^-1 for a small library with 3 x 10^6 clones (Griffiths et al., 1993; Krebs et al., 1998; Griep et al., 1999; Villani et al., 2005) to 10^8 to 10^10 M^-1 for a very large repertoire with 10^10 to 10^12 clones (Eeckhout et al., 2004).

An alternative approach is the creation of synthetic or semi-synthetic human antibody V-gene repertoires by the in vitro assembly of V-gene segments (De Kruijf et al., 1995; Griffiths et al., 1994). The folding and specificity of antibody fragments can be optimized by modulating the framework regions and CDRs (Knappik and Plückthun, 1995), e.g. by random in vitro mutation using error-prone PCR (Martineau, 2002), site-directed mutagenesis (Winkler et al., 2000) or by molecular evolution (Patten et al., 1997; Proba et al., 1998).

Libraries can also be tailored to select for antibodies with useful characteristics, such as stability in different intracellular compartments. For example, it has been shown that less than 1% of scFvs are stable when expressed in the plant cytosol and only 0.1% are both stable and functional (Auf der Maur et al., 2004; Visintin et al., 2004). Libraries of scFvs that are pre-selected for cytosolic stability can be constructed by focusing on a single framework (Philibert et al., 2007) or a small number of frameworks that are known to be stable in this environment (Auf der Maur et al., 2002; Lee et al., 2004). Such antibody libraries have been used to generate stable scFvs that bind to Cucumber mosaic virus (CMV) (Villani et al., 2005) and Potato virus X (PVX) (Villani et al., 2008).

Alternative display technologies have been developed using ribosomes or mRNA instead of bacteriophages, allowing the construction of highly complex libraries that facilitate in vitro evolution through iterative rounds of mutagenesis and selection (Hoogenboom et al., 1991; Yan and Xu, 2006). Ribosome display is a cell-free system for the selection of proteins and peptides from large libraries, which involves the formation of stable protein–ribosome–mRNA (PRM) complexes that are panned against the antigen. The selected PRM complexes are then reverse transcribed to cDNA and their sequences are amplified via PCR (Hanes and Plückthun, 1997; He and Taussig, 2007; Shibasaki, 2009). Similarly, mRNA display uses a complex of mRNA and the encoded polypeptide as the basis for selection although in this case they are linked by puromycin (Takahashi et al., 2003; Tugores and Izipisua Belmonte, 2008). During panning, mRNA–protein fusions that bind well to the antigen are reverse transcribed and amplified, generating a PCR product encoding a high-affinity peptide. The mRNA–protein complex is generated entirely in vitro which allows the construction of highly complex libraries typically containing 10^13–10^15 unique sequences (Keefe and Szostak, 2001; Xu et al., 2002; Takahashi et al., 2003).
4. Antibody-based resistance against plant viruses

The concept of ectopic antibody expression was first established in mammalian cells, where the intracellular antibodies were described as ‘intrabodies’ (Baltimore, 1988) and were developed as drugs designed to interrupt the HIV-1 infection cycle (Marasco, 1995). Recombinant antibodies were first expressed in plants by Hiatt et al. (1989) using a stacking approach in which plant lines individually expressing the heavy and light chains were crossed to produce double transgenics expressing full-size assembled antibodies. Several groups then attempted to use plant-derived antibodies (sometimes described as ‘plantibodies’) to interrupt the infection cycle of plant viruses, which went hand in hand with basic research to investigate plant disease mechanisms (Cervera et al., 2010; Fischer et al., 2001; Prins et al., 2008; Safarnejad et al., 2009; Schillberg et al., 2001; Tavladoraki et al., 1993; Twyman et al., 1997; Voss et al., 1995; Zimmermann et al., 2006). Antibodies and their fragments can neutralize viruses by preventing uptake, or by disrupting replication, assembly, local movement and systemic spreading (Prins et al., 2008; Turturo et al., 2008). The impact of virus-specific recombinant antibodies was found to depend on three main factors: quantity (yield and stability), localization (intracellular targeting) and binding efficiency (affinity and avidity).

Plant viruses are biotrophic parasites, i.e. they are not considered as living organisms outside the plant cell environment. Therefore the life cycle begins with the uptake of virions into the cytosol of a plant cell, through a wound caused by mechanical damage or an insect vector (Fig. 2). Inside the plant cell, the virus disassembles (partial or complete removal of the capsid) and the virus genome is expressed, relying on from DNA genomes) and also for transcription in the case of DNA viruses. Therefore, despite the suboptimal environment for antibody folding and assembly, the cytosol of plant cells is still suitable as a target for antibodies intended to provide disease resistance in plants.

The achievements described above for TMV have been confirmed with other plant viruses. For example, Villani et al. (2005) expressed two scFv fragments with high affinity for the CMV coat protein in Nicotiana benthamiana plants, showing they could accumulate to high levels. The scFvs were originally derived from a semi-synthetic phage display library built around a thermodynamically-stable single framework scaffold. Transgenic tomato plants were then produced expressing the antibodies in the cytosol, and a challenge with CMV showed that plants expressing either antibody were able to suppress the infection. Further selection resulted in the identification of transgenic line that was fully resistant to the virus.

In another study, a scFv with high affinity for the CP21 major coat protein of Beet necrotic yellow vein virus (BNYVV) was isolated by panning an immunized phage display library (Jahromi et al., 2009). Transgenic sugar beet plants accumulating the cytosolic scFv showed strong resistance against the virus (Malboobi and Arabi, unpublished data).

The endoplasmic reticulum (ER) of plant cells also provides a beneficial environment for protein folding and assembly, and the retention of antibodies in the ER (using a C-terminal KDEL tag on one or both chains) can help to maintain their affinity and specificity (Fecker et al., 1997). However, the ER is generally not the best compartment to achieve virus contact. Hence, the ER-retention of a scFv recognizing the coat protein of BNYVV in transgenic N. benthamiana plants achieved only partial protection against the virus (Fecker et al., 1997).

The presence of conserved domains within virus coat proteins could lead to the development of antibodies that protect plants against multiple viruses. One example is the monoclonal antibody 3D–17, which was raised against Johnson grass mosaic virus (JMV) but recognizes 14 other potyviruses. This has been used to generate a scFv with the potential to protect tobacco plants against at least these 15 potyvirus pathogens and possibly others (Xiao et al., 2000). The cytosolic accumulation of the recombinant antibody was negligible in transgenic plants, but the secreted form accumulated in the range 0.006–0.02% TSP and provided partial protection against challenges with Potato virus Y strain D (PYY-D) and Clover yellow vein virus (CYVV) (Xiao et al., 2000). Similarly, Nölke et al. (2009) generated a monoclonal antibody FL3 recognizing the coat protein of Grapevine fanleaf virus (GFLV), but the antibody was also able to bind Arabis mosaic virus (ArMV) which contains conserved epitopes. A recombinant antibody fragment (scFvGFLVcp-55) derived from FL3 was expressed in transgenic N. benthamiana plants, where it accumulated in the cytosol in the range 0.002%–0.1% TSP. Challenge studies in T1 and T2 plants infected with GFLV or ArMV showed that complete resistance could be achieved against GFLV when the recombinant antibody accumulated to 0.05% TSP (Nölke et al., 2009).

Coat proteins are not the only structural proteins that can be targeted by recombinant antibodies. Tomato spotted wilt virus (TSWV) contains
three additional structural proteins: the N protein which binds to the genomic RNA, and two G proteins which are associated with the viral envelope. Transgenic plants expressing high levels of a cytosolic scFv recognizing the N protein were resistant to the virus, but the accumulation of G protein-specific scFvs in the ER (where the virus accumulates prior to acquisition by an insect vector) did not inhibit virus transmission (Prins et al., 2005).

Recently, antibody-mediated virus resistance has been demonstrated in citrus trees which are susceptible to the devastating Citrus tristeza virus (CTV) (Cervera et al., 2010). Monoclonal antibodies 3CA5 and 3DF1, which bind specifically to the CTV p25 major coat protein, were used to generate scFv fragments with the same specificity. Constitutive cytosolic expression of these recombinant antibodies, individually and in combination, was tested in transgenic Mexican lime plants (Citrus aurantifolia). Although the antibodies accumulated to barely detectable levels, there was nevertheless a clear protective effect in most transgenic lines following a challenge with CTV induced by graft inoculation. Overall, 40–60% of the plants were resistant, as revealed by the absence of virus antigens in tissue-print ELISAs, and those with the highest levels of antibody in western blot assays were the most resistant. The parental 3CA5 and 3DF1 antibodies recognize all the diverse CTV strains isolated thus far, so the resistance achieved in the experimental transgenic plants should be widely applicable in the field. The p25 protein is required for virus assembly, spreading and transmission as well as the suppression of intercellular silencing, so resistance phenotypes in transgenic Mexican lime plants involve the interruption of the virus life cycle at multiple stages (Cervera et al., 2010).

4.2. Antibodies that bind non-structural virus proteins

The large amount of virus coat protein that accumulates in plant cells during an infection may prevent antibodies expressed at very low levels from achieving complete neutralization. The successes achieved with coat protein-specific antibodies may therefore reflect...
the ability of antibodies to bind the incoming virus particles, interfering with uncoating and the early steps of replication, when the number of potential targets is still small. An alternative approach is the expression of recombinant antibodies recognizing non-structural proteins that are necessary for virus replication or movement, since these proteins do not accumulate in such large amounts.

The first such report involved the creation of scFv2A, which bound to RNA replicate Nl from Plum pox virus (PPV). The scFv was made by modifying the pre-existing hybridoma clone, mAb2A. Transient expression in N. benthamiana revealed low-level accumulation of functional scFv2A in the cytosol as determined by ELISA (Esteban et al., 2003). Transgenic N. benthamiana plants were then generated with the scFv targeted to different compartments such as the cytosol, ER and nucleus, and challenge studies revealed that infections were suppressed wherever the antibody accumulated (Gil et al., in press).

RNA viruses require RNA-dependent RNA polymerases (RdRps) for replication. These enzymes are present in very small amounts and contain several conserved motifs that are suitable antibody targets. Boonrod et al. (2004) expressed cytosolic and ER-localized scFvs recognizing a conserved domain of the Tomato bushy stunt virus (TBSV) RdRp in N. benthamiana, and the transgenic plants showed varying levels of resistance not only against TBSV, but also Cucumber necrosis virus (CNV), Turnip crinkle virus (TCV) and Red clover necrotic mosaic virus (RCNMV), which represent different genera of two plant virus families. Complete resistance against TBSV was achieved when the scFv reached 0.01% TSP (Boonrod et al., 2004). Similarly, Nickel et al. (2008) expressed a scFv derived from the full-size monoclonal antibody P1-1, which binds specifically to the C-terminal portion of the Potato leaf roll virus (PLRV) P1 protein. This is required for the maturation of the genome-linked protein VPg, which must be present in its mature form to facilitate virion assembly. Both transient and stable expression of scFvP1-1 in plants reduced the accumulation of virus particles and therefore achieved a high level of resistance against PLRV. Fomitcheva et al. (2005) extended the principle of broad-spectrum resistance by isolating a scFv that bound to the highly-conserved glycine–aspartate–aspartate (GDD) motif in the active site of RdRp from positive-strand RNA plant viruses. The scFv recognized RdRps from numerous viruses in several different families in vitro, but has yet to be tested in transgenic plants.

Potyviruses such as PVY synthesize a major polyprotein during the early part of the infection cycle, and this must undergo proteolytic processing (carried out by the viral protein Nla) to produce mature functional proteins. A scFv that binds specifically to Nla was expressed in two transgenic potato cultivars (Claustar and BF15) and accumulated to levels that were sufficient to provide complete protection against a challenge with PVY (Gargouri-Bouzid et al., 2006). Similarly, Bouaziz et al. (2009) expressed a single-domain camelid nanobody against Nla, and its accumulation in the cytosol was sufficient to attenuate the symptoms of PVY infection.

Plant viruses generally encode a movement protein which interacts with microtubules and plasmodesmata to facilitate cell-to-cell transport, thus spreading the infection to a local group of cells. Movement proteins are therefore promising targets for recombinant antibodies because the absence of a functional movement protein prevents viruses spreading beyond the initial infected cell. Zhang et al. (2008) generated nine scFvs recognizing conserved domains in TSWV movement protein (NSm) and expressed these antibodies in the cytosol of transgenic tobacco plants. Two of the scFvs were expressed at high levels in the cytosol (5.9–8% TSP) and achieved a significant delay in the onset of disease symptoms.

Most antibodies designed to achieve disease resistance in plants have been developed against RNA viruses, but Safarnejad et al. (2008) have extended the principle to Tomato yellow leaf curl virus (TYLCV), a geminivirus with a circular ssDNA genome. They generated two scFvs (ScRep1 and ScRep2) recognizing different epitopes of the major replication-associated protein (Rep) by panning naïve and immunized phage display libraries. Serological analysis showed that the antibodies recognized different Rep domains essential for virus replication. Both ScRep1 and ScRep2 accumulated to high levels in the cytosol of infiltrated tobacco plants, and analysis of crude leaf extracts confirmed that the antibodies were able to bind their target antigen with high affinity (Safarnejad et al., 2008). A ScRep1 fusion with green fluorescent protein (GFP) reduced the accumulation of virus DNA in transgenic plants, the first demonstration of antibody-mediated resistance involving a DNA virus (Safarnejad et al., 2009).

5. Antibody-based resistance against bacterial pathogens

A number of strategies have been developed to achieve resistance against bacterial diseases in plants, such as disrupting bacterial pathogenicity, augmenting natural defense systems, expressing heterologous antimicrobial proteins, and inducing cell death at the site of infection by promoting the hypersensitive response (Loebenstein, 2009; Mourguès et al., 1998). Recombinant antibodies were first used as a strategy to control bacterial infections by Le Gall et al. (1998). They generated a scFv fragment recognizing the major immunodominant membrane protein (IMP) of stolbur phytoplasma, a mollicute that is mainly restricted to the phloem sieve elements of infected hosts. The scFv was derived from the full-size monoclonal antibody 2A10, originally isolated from a hybridoma line, and was expressed in E. coli to confirm its specificity and stability. The scFv-2A10 was then constitutively expressed in the cytosol of transgenic tobacco plants using the CaMV 35S promoter, which is active in the phloem among many other tissues. Transgenic scions grafted onto infected tobacco rootstock remained symptomless and flowered after 2 months, whereas control scions from wild type plants were infected by the rootstock and died before flowering.

A complementary study was performed using the same scFv to compare cytosolic expression with secretion to the apoplast (Malembic-Maher et al., 2005). The scFv accumulated to 0.04% TSP in the apoplast whereas cytosolic accumulation was below the detection threshold. Grafting studies were carried out using infected rootstock both in the greenhouse and in the field. In contrast to the earlier report, cytosolic expression of the scFv had no impact on the pathogen, whereas secretion to the apoplast delayed the onset of symptoms by approximately 2 weeks and the symptoms were less severe (Malembic-Maher et al., 2005).

A scFv recognizing maize stunt spiroplasma (CSS) was derived from a full-size monoclonal antibody with the same specificity, and was expressed in the cytosol of transgenic maize plants using the constitutive maize ubiquitin-1 (Ubi-1) promoter. Although the scFv could be detected in transgenic plants, there was no effect on disease progression when the plants were challenged with CSS (Chen and Chen, 1998).

6. Antibody-based resistance against fungal pathogens

The investigation of fungal pathogens and their interactions with plants has identified several genes that confer resistance against pathogenic fungi. These encode a variety of products, including antimicrobial peptides, enzymes that synthesize antifungal metabolites, growth inhibitors, proteins that inhibit fungal virulence proteins, and proteins that induce natural plant defenses including the hypersensitive response (Punja, 2001). Some of these genes have been expressed in plants to achieve disease resistance (Billesfeld et al., 1999; Osusky et al., 2000; Punja, 2001; Shah, 1997; Wally et al., 2009).

Antibodies were first investigated as a strategy to prevent fungal diseases by Peschen et al. (2004). They expressed a fusion protein comprising a recombinant scFv recognizing a surface protein present on Fusarium oxysporum f. sp. matthiae joined to an antifungal protein from Aspergillus giganteus. The scFv was isolated from a phage display library derived from...
chickens immunized with fungal spores. Preliminary analysis confirmed that the scFv-AP fusion bound to the fungus in vitro and inhibited its growth. Transgenic Arabidopsis thaliana plants expressing the fusion protein showed a high level of resistance when challenged with the pathogen, and were protected more robustly than plants expressing either the recombinant antibody or the antifungal peptide alone (Peschen et al., 2004).

A complementary study was reported by Li et al. (2008) in which the same fusion protein was expressed in transgenic wheat. The transgenic plants showed strong resistance when challenged by inoculation with Fusarium asiaticum microconidia, and the yield of grain in the transgenic plants was up to 4.7-fold higher than in control plants. The resistance phenotype was maintained in subsequent generations.

Recently, Yajima et al. (2010) expressed a scFv recognizing the pathogen Sclerotinia sclerotiorum in rapeseed plants (Brassica napus). The scFv was isolated from a phage display library prepared from the spleen cells of pre-immunized mice (Yajima et al., 2008). Transgenic plants expressing the scFv (confirmed by RT-PCR) showed partial resistance to S. sclerotiorum, with less severe symptoms that wild type plants (fewer and less extensive necrotic lesions) even though the scFv could not be detected by western blot (Yajima et al., 2010).

7. Additional approaches

Antibody stacking, i.e. the simultaneous expression in transgenic plants of two or more different scFvs with different target specificities, can be used to achieve broad-spectrum and durable resistance (Schillberg et al., 2001). This can be achieved by expressing multiple scFvs from different genes, but a simpler approach is to construct diabodies or dimerized antibodies because multivalent antibodies have greater avidity and can bind specifically to a unique range of pathogens. Fischer et al. (2001) described the production and expression of a bispecific recombinant antibody (biscFv2429) in tobacco suspension culture cells and transgenic tobacco plants. The molecule was a combination of the scFv24 and scFv29 recombinant antibody fragments which bind respectively to neotopes of intact TMV virions and a cryptotope of the TMV coat protein monomer. The two fragments were joined using the Trichoderma reesi cellobiohydrolase I linker. The biscFv2429 was targeted to different cell compartments such as the cytosol (low-level accumulation), apoplast and ER (both high-level accumulation). The bispecificity and bivalency of the recombinant fragment were confirmed by ELISA and surface plasmon resonance spectroscopy.

In the vertebrate immune system, antibodies are present as both diffusible proteins and cell-surface receptors on B-lymphocytes. This can be mimicked in plants by expressing antibodies with an integral transmembrane domain, so they are inserted in the plasma membrane and gain the ability to confront pathogens at the cell surface. Schillberg et al. (2000) have shown that such membrane-anchored antibodies are able to confer resistance against plant viruses by expressing in transgenic tobacco plants and suspension cells a re-engineered version of the TMV-specific fragment scFv24, incorporating a mammalian signal peptide and the trans-membrane domain from a membrane-spanning receptor. The signal peptide ensured secretion, but the trans-membrane domain trapped the recombinant antibody in the plasma membrane with the antigen-binding domain on the outside of the cell. Serological analysis and immunolocalization followed by electron microscopy confirmed the recombinant antibody was expressed and targeted to the plasma membrane as anticipated. Transgenic plants expressing the membrane-bound scFv were protected against a challenge with TMV (Schillberg et al., 2000).

8. Optimizing yields and achieving appropriate sub-cellular targeting

As stated earlier, antibodies are much more effective against plant pathogens when expressed at high levels, and strategies to achieve this include the design of optimized expression cassettes, the incorporation of powerful promoters and the improvement of protein stability and accumulation in cells through the use of appropriate sub-cellular targeting signals (Desai et al., 2010).

Promoter choice is important because it determines the amount of mRNA available for translation and also the cell types in which this mRNA accumulates. Constitutive, inducible and tissue-specific promoters are available, but in most cases antibodies have been expressed using the constitutive and highly active CaMV 35S promoter for dicotyledonous plants and the maize ubiquitin-1 and rice actin-1 promoters for monocotyledonous plants (Christensen and Quail 1996; Li et al., 2008; Nickel et al., 2008; Safarnejad et al., 2009; Schillberg et al., 1999). The CaMV 35S promoter results in very high levels of transcription in most dicotyledonous plant cells (Benfey et al. 1990).

In addition to constitutive promoters, spatiotemporal (tissue or stage-specific) promoters can be used to restrict expression to different plant tissues (Peremarti et al., 2010). These promoters are potentially useful for plant pathogens that attack specific tissues, e.g. roots for nematodes and soil-borne pathogens, phloem for phytoplasmas, or leaves and stems for rusts, because this reduces the resources committed by the plant to the expression of a recombinant protein in tissues where it would not be required, and also reduces the perceived risks of genetically modified food crops by allowing the exclusion of recombinant proteins from harvested tissues (Bergmans et al., 2008; Ghasimi et al., 2009; Moon et al., 2010). To the best of our knowledge, spatiotemporal promoters have yet to be used for recombinant antibody expression as a disease resistance strategy, but the feasibility of leaf-specific promoters such as rhs and spas1 and tuber-specific promoters such as patatin have been demonstrated (Chavez-Barrenas et al., 2000; Park and Cheong, 2002; Salehi Jouzani et al., 2008). Zhao et al. (2004) showed that the phloem-specific AtSLC2 promoter, which controls a sucrose transporter gene, is suitable for the development of phytoplasma-resistant transgenic strawberries. Inducible promoters could also be useful for antibody-mediated disease resistance if the antibody gene could be induced by the presence of the pathogen. Although most reports of inducible promoter use for antibody expression have focused on molecular farming in cultured plant cells (Doli et al., 2006; Franconi et al., 2010; Huang et al., 2009; 2010; Mavittuna, 2005; Shin et al. 2003), the wound-inducible MeGA promoter (Hansen et al., 1997) and HMG2 promoter (Cramer and Weissenborn 1997) could be valuable to induce responses against virus pathogens borne by insect vectors.

Effective antibody-mediated disease resistance requires some knowledge of the infection cycle to ensure that the recombinant antibody is targeted to a sub-cellular compartment where it will encounter the pathogen. In the case of viruses, most of the essential steps take place in the cytosol (Baulcombe, 1994). The recombinant antibody must therefore accumulate in the cytosol even though the reducing environment and lack of chaperones discourage correct folding and assembly, making it difficult to achieve high expression levels (Fecker et al., 1996). Many researchers have reported barely detectable expression levels for scFvs in the cytosol of plant cells (Conrad and Fiedler, 1998; De Jaeger et al., 1999; Fischer et al., 2001), but others have reported high accumulation levels, suggesting that certain scFvs have framework regions that contain important determinants of folding efficiency in the cytosol (Safarnejad et al., 2009; Zhang et al., 2008).

Recombinant proteins fold and assemble much more efficiently in alternative compartments such as the apoplast and ER (De Jaeger et al., 2000; Lessard et al., 2002; Mavittuna, 2005) because the secretory pathway provides an oxidizing environment, contains molecular chaperones and lacks proteases (Conrad and Fiedler, 1998; Schillberg et al., 1999; Schillberg et al., 2001). Although the cytosol is most appropriate for encountering viruses, the apoplast is arguably better for encountering fungi because these pathogens are most vulnerable when they are still outside the cell (Li et al., 2008; Schillberg et al., 1999).

The inclusion of a C-terminal KDEL signal retrieves secreted antibodies to the ER, which can increase yields by 10–100-fold compared with expression in the cytosol.
to the apoplasm (Conrad and Fiedler, 1998). Interestingly, the addition of a KDEL signal without an accompanying N-terminal signal peptide can stabilize the increase in stability of the proteins in the cytosol by inhibiting pro tease activity (Schouten et al., 1996).

9. Conclusions

Plant diseases caused by viruses, bacteria, fungi and nematodes are recognized as one of the most severe constraints affecting agricultural productivity. Conventional breeding can be used to introgress natural resistance genes into commercial cultivars, but only if resistance genes are available in sexually-compatible species. Genetic engineering can overcome the species barriers of conventional breeding and is therefore the most promising strategy to optimize crop yields and quality. Antibody-mediated resistance is a recent innovation in agriculture which can be used as an alternative to, or in combination with, conventional breeding to boost yields. At the same time, antibodies binding to pathogen proteins provide a useful approach for the investigation of plant diseases and plant–pathogen interactions.

Antibodies (or antibody fragments such as scFvs) that bind pathogen proteins with high affinity can be generated rapidly using phage display and related display technologies. These can be used to select scFvs recognizing any conceivable antigen, even those that are not suitable for immunization. Variation can be introduced using molecular evolution technologies, and the selection pressure imposed during library panning then helps to enrich the library for scFvs that bind pathogen antigens with high affinity. Antibodies recognizing conserved domains can achieve resistance against multiple pathogens at the same time.

Despite the promise of recombinant antibodies and the major proof-of-principle achievements thus far, this approach has not been widely adopted and no antibody-based disease resistance traits are in commercial development. This may reflect the multidisciplinary nature of the approach, which requires expertise in immunology in addition to plant pathology and genetic engineering. There may also be some concerns about the safety of mammalian proteins (and recombinant versions thereof) expressed in food crops.

There are several challenges that remain to be addressed, including the optimization of cytosolic antibody accumulation. The reducing environment, coupled with the presence of proteases and lack of chaperones can be overcome by the inclusion of protein tags that resist proteases and the use of frameworks that are cytosol-tolerant, which can be achieved using appropriate selection pressure to enrich libraries for stable antibodies. An alternative approach is the use of single-domain antibodies from camellias, which require only the heavy chain variable region to bind antigens efficiently. Innovative approaches such as the use of camellia antibodies, bispecific antibodies, and antibodies targeted to the plasma membrane and other sub-cellular compartments, provide fertile ground for further research and the development of high-yielding crops with long-lasting resistance against the most prevalent and destructive crop diseases.

References


Marasco WA. Intracellular antibodies (intrabodies) as research reagents and therapeuti-}


Tillib S. “Cameleoantibody” is an efficient tool for research, diagnostics and therapy. Mol Biol 2011;45:66–73.


