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Phage display-selected single-chain antibodies confer high levels of resistance against *Tomato spotted wilt virus*

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Rational design of antibodies targeting essential viral proteins can complement the palette of antiviral resistance strategies. Here, stable and high expression of single-chain monoclonal antibodies targeting the nucleoprotein of the economically important plant virus *Tomato spotted wilt virus*, a protein that is involved in multiple steps in the viral infection cycle, is reported. High cytoplasmic expression levels of three selected phage display-derived anti-viral single-chain antibodies were established. Of these antibodies, two led to high levels of resistance against this plant virus. Protoplast experiments provided evidence that the two resistance-conferring antibodies may have a different mode of action and could be combined for higher durability of resistance in the field.

INTRODUCTION

Natural sources of resistance against plant pathogens are limited and cannot be renewed once the pathogen has found a way to overcome the resistance. Therefore, great attention has been given to renewable forms of engineered resistance. For resistance against viruses, for example, RNA silencing has proved very successful over the years, especially when using inverted repeat constructs (Prins & Goldbach, 1996; Baulcombe, 1999; Smith *et al.*, 2000). Transgenic expression of antibody (fragments) holds the promise of being one of those strategies. However, since the serendipitous first report on this subject (Tavladoraki *et al.*, 1993), it has taken over a decade of painstaking technology development before resistance levels were improved for another virus, *Tomato bushy stunt virus* (TBSV), also of the plus-strand genus *Tombusvirus* (Boonrod *et al.*, 2004).

In the archetypical plantibody resistance paper (Tavladoraki *et al.*, 1993), the authors demonstrated reduced virus accumulation and delay of symptom expression in plants expressing a hybridoma-derived single-chain variable antibody fragment (scFv) targeted against the coat protein of the tombusvirus *Artichoke mottled crinkle virus*. The mechanism by which the antibody conferred this partial resistance remained unsolved. Similarly, delay and suppression of virus symptoms were later observed against *Beet necrotic yellow vein virus* (Fecker *et al.*, 1996) and *Tobacco mosaic virus* (Schillberg *et al.*, 2000). These studies used either full-sized or recombined fragments of antibodies from hybridoma cell lines. As an alternative, the expression of antibody fragments in a phage display format (e.g. Vaughan *et al.*, 1996) permits rapid antibody cloning and gives great flexibility in selecting and modifying specific antibodies and, in the case of synthetic libraries, avoids the use of animals. Several successful examples of the use of phage display technology for the generation of virus-specific antibodies have been described (Ziegler *et al.*, 1995; Fecker *et al.*, 1996; Harper *et al.*, 1997; Boonham & Barker, 1998; Griep *et al.*, 2000). Expression levels of single-chain antibodies are generally high when proteins are excreted into the apoplast by including signal sequences to the transformation construct (Owen *et al.*, 1992), and even higher when retained in the endoplasmic reticulum (ER) by the C-terminal addition of the KDEL retention tetrapeptide (Conrad & Fiedler, 1998). However, expression of scFvs in the cytoplasm of plants, where the vast majority of plant viruses replicate, has long been troublesome. In an excellent recent paper, Boonrod *et al.* (2004) selectively developed single-chain antibody fragments targeting the conserved replicase of TBSV.

The negative-strand *Tospoviruses* were the subject of this study. These viruses form a distinct genus of phytopathogenic viruses within the arthropod-borne family *Bunyaviridae* (Elliott *et al.*, 2000), which is further restricted to animals. The genome of *Tomato spotted wilt virus* (TSWV), economically the most important tospovirus, consists of three RNA species (de Haan *et al.*, 1990, 1991; Kormelink *et al.*, 1992). The five viral open reading frames specify a total of six mature viral proteins. These are the nucleoprotein (N); two envelope glycoproteins (G1 and G2); the viral polymerase (L); and two non-structural proteins, NS_M,

Fig. 3 is available in colour as supplementary material in JGV Online.

the viral movement protein (Storms *et al.*, 1995), and NS_S, which is involved in suppression of RNA silencing (Bucher *et al.*, 2003). In natural infections, tospoviruses enter the plant cell during probing or feeding by viruliferous thrips. Upon entering the cell, the virus is relieved of its membrane and infectious nucleocapsids are released into the cytoplasm. At this stage, the viral RNA will be either transcribed or replicated. The transcription-to-replication switch is thought to be controlled by the concentration of free N protein in the cytoplasm. At low N protein concentrations, i.e. at the onset of infection, the replicase will produce viral mRNAs, by means of cap snatching (Duijsings *et al.*, 2001). Following translation of the N protein and its increased concentration, the polymerase switches to replicase mode at which time viral genomic RNAs are multiplied and associate with the N protein into nucleocapsids (Prins & Goldbach, 1998). The viral replicase is important in these processes, but the N protein also plays a key role. In addition, this protein is involved in several later processes such as viral RNA packaging into nucleocapsids, cell-to-cell movement to neighbouring cells through virus-induced tubules (Storms *et al.*, 1995) and the formation of new virus particles by associating with viral glycoproteins at the Golgi membranes (Kikkert *et al.*, 1999). Early steps in the tospovirus replication cycle in plants are prime candidates for targeting by protective plant-expressed antibodies, as in these conditions the stoichiometry of the antibody versus its target antigen is most favourable. Hence the switch between replication and transcription, as well as both processes *per se*, are the Achilles heel of a successful virus infection and the target of the approach used here.

In our previous work (Griep *et al.*, 2000), we isolated and characterized 12 scFv clones targeting the TSWV N gene product. These scFvs were derived from a phage display library (Vaughan *et al.*, 1996). Four of these scFv clones (N3, N19, N56 and N97) not only cross-reacted with TSWV, but also reacted with the related tospoviruses *Tomato chlorotic spot virus* (TCSV) and *Groundnut ringspot virus* (GRSV). As these monoclonal single-chain antibodies also recognize other tospoviruses, we reasoned that they might target conserved, essential epitopes within the N protein, making them excellent candidates for conferring resistance to transgenic plants.

In this study, we aspired to combine virological knowledge on the infection cycle of tospoviruses in plants with optimized cytosolic stability of scFvs. This was achieved using scFvs derived from a phage display library and by adding the KDEL tetrapeptide. Transgenic plants expressing these scFvs were produced and tested for virus resistance.

METHODS

Viruses and plants. TSWV strain BR-01 (de Ávila *et al.*, 1993) was maintained on *Nicotiana benthamiana*. Recipient plants used in the transformation experiments were *N. benthamiana* plants. All manipulations with transgenic plant material were carried out under conditions (PKII) specified by the Dutch authorities (VROM/COGEM, GGO 97-150).

Transformation of tobacco. All TSWV sequences containing pBIN19-derived vectors were introduced into *Agrobacterium tumefaciens* strain LB4404 (Ditta *et al.*, 1980) by triparental mating, using pRK2013 (Horsch *et al.*, 1985) as a helper plasmid. *N. benthamiana* plants were transformed and regenerated as described by Horsch *et al.* (1985).

Western blot analysis of scFv expression in transgenic plants. Transgenic plant lines were checked for expression levels of transgenic scFvs by Western blot analysis of leaf material. For this purpose, leaf disks of transgenic plants collected prior to virus inoculation were ground in an equal volume of PBS and boiled in dithiothreitol (DTT)-containing protein-loading buffer (50 mM Tris/HCl pH 6·8, 100 mM DTT, 2 % SDS, 10 % glycerol, 0·01 % bromophenol blue). Proteins were separated on a 12 % polyacrylamide gel and transferred to Immobilon membranes. Membranes were blocked overnight in 3 % skimmed milk powder in PBS. Transgenic (scFv) protein production could be monitored due to the C-terminal addition of the cMyc epitope. This epitope was recognized using the 9E10 monoclonal antibody (Munro & Pelham, 1986). Goat anti-mouse antibodies conjugated to alkaline phosphatase were applied in the second-round incubation; visualization was done by the addition of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Tospovirus resistance assays in plants and protoplasts. Inoculation of tospoviruses and ELISA tests were performed using standard conditions as first described by Gielen *et al.* (1991). Isolation of protoplasts and inoculation with TSWV were according to Prins *et al.* (1997).

RESULTS

Selection of scFvs, cloning and transformation of plants

Three scFv clones recognizing the TSWV N gene (N3, N56 and N97; Griep *et al.*, 2000) were selected for further cloning and eventual expression in plants. The pRAP vector (Fig. 1) was previously designed (Schouten *et al.*, 1997) to clone phage display-derived scFv cDNAs in such a way that the resulting proteins are fused to a signal peptide at the N terminus and to a *cMyc* tag at the C terminus directly followed by the KDEL tetrapeptide. For appropriate functioning of anti-TSWV antibodies, it is imperative that these are targeted to the cellular compartment in which the virus replicates, i.e. the cytoplasm. Hence, the scFvs were cloned into pRAP using appropriate restriction sites, such that the ER targeting signal sequence was removed (Fig. 1). To enhance stability and therefore expression levels of the scFvs in the cytoplasm (Schouten *et al.*, 1997), the KDEL tetrapeptide was retained as a C-terminal translational fusion. The pRAP vector also supplies the scFvs with the enhanced *Cauliflower mosaic virus* 35S promoter and *nos* terminator. Finally, all sequenced expression constructs were cloned into the binary vector pBINPLUS (van Engelen *et al.*, 1994) (Fig. 1). The three N-targeting pRAP constructs were transformed into *N. benthamiana* using *A. tumefaciens*; this resulted in 23 transgenic plants. Plants were grown to

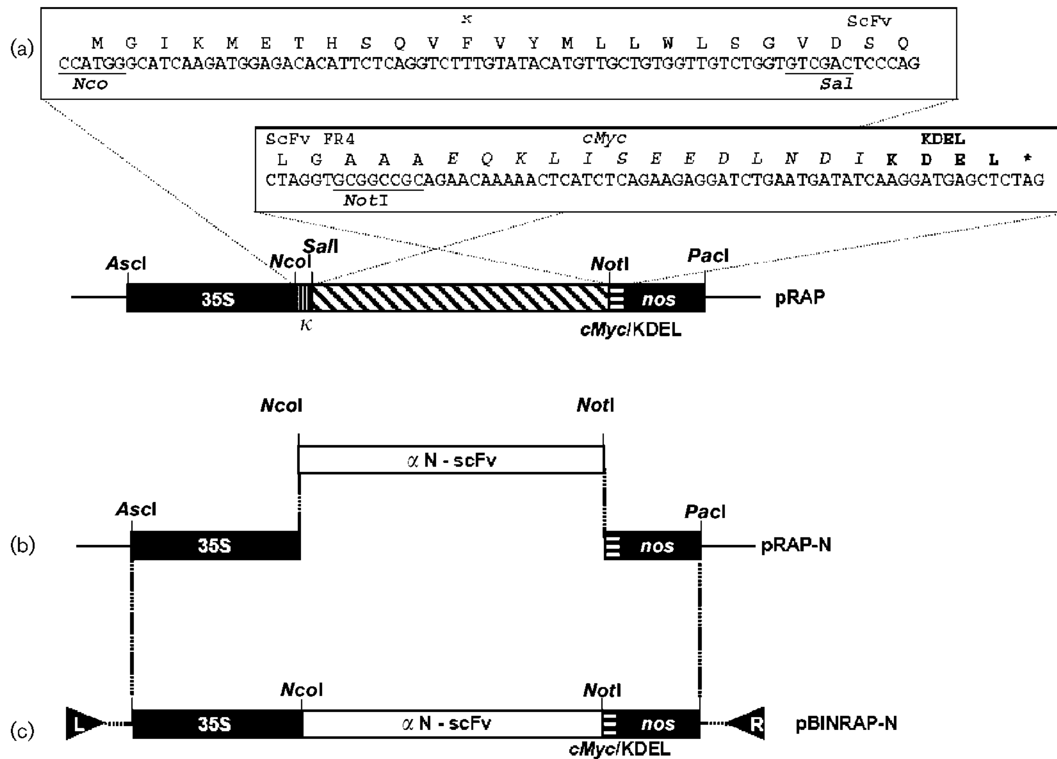

Fig. 1. Cloning scheme of the previously isolated scFv fragment genes targeting the TSWV N gene into the pBINRAP vector system. (a) The pRAP vector-supplied scFv cDNAs from a phage display library with the coding sequences of the murine signal sequence (κ) (N-terminal) and a C-terminal *cMyc* tag (for detection) and KDEL (for cytoplasmic stability). (b) Using the *Nco*I and *Not*I sites for cDNA cloning, *cMyc* and KDEL were retained. The κ signal sequence, however, was removed. The pRAP vector also supplied the scFv with 35S promoter sequences and a *nos* terminator. (c) Final cloning between left (L) and right (R) borders of pBINPLUS was performed using *Asc*I and *Pac*I sites and yielded the plant transformation vector pBINRAP-N that was subsequently used for *Agrobacterium*-mediated plant transformation.

maturity and R_1 seeds were collected (Table 1). Self-fertilized R_1 seeds were germinated on kanamycin-containing medium and used for molecular analysis and virus inoculation assays.

ScFvs accumulate to high levels in transgenic plants

Our previous research indicated that high expression levels of phage display-derived scFvs could be reached in transgenic plants with the addition of the KDEL peptide (Schouten *et al.*, 1997). We aspired to use this knowledge to optimize the expression of scFv proteins against TSWV. Although the expressed amount of protein varied among the lines (Fig. 2), the scFv expression levels in most plants were estimated to be between 0·2 and 0·8 % of the total soluble protein, as deduced from Western blot analysis and comparative ELISA. ScFvs of all three types tested (N3, N56 and N97) exhibited similar distributions of expression level of which Fig. 2 is a typical example. High expression levels were observed in several plants for all three constructs used. This confirmed the notion that high expression levels of phage display-derived antibody fragments in the plant cytosol can be reached by cloning phage display-selected single-chain antibodies fused translationally to the KDEL tetrapeptide.

Resistance to TSWV is restricted to high expressers of single chains N3 and N56

Twenty-three self-fertilized (R_1) lines of transgenic *N. benthamiana* plants expressing anti-N scFv were tested for resistance against inoculation with TSWV. In each inoculation experiment, 10 plants were challenged with the virus. As indicated in Table 1, plants of seven lines expressing N3 or N56 single-chain fragments showed a marked resistance against the virus. These plants completely lacked symptoms of viral infection, both in inoculated and in systemic leaves (Fig. 3) throughout the experiment up to flowering and seed setting (maximum 60 days post-inoculation). Non-transgenic control plants, as well as susceptible transgenic plants, indiscriminately developed local symptoms after 2–3 days and systemic symptoms between 3 and 5 days post-inoculation. The lack of visible symptoms on both inoculated and systemic leaves correlated with the lack of TSWV N protein in ELISA assays at 7 days post-inoculation

Table 1. Resistance against TSWV in transgenic *N. benthamiana* plant lines expressing anti-TSWV scFvs

NT, Not tested.

ScFv clone	Line	Resistance in R_1 plants*	Resistance in R_2 plants*†
N3	1	8/10	5/5 (4)
	2	6/10	5/5 (4)
	3	0/10	–
	5	0/10	–
	6	8/10	5/5 (4)
	8	0/10	–
	11	0/10	–
	13	7/10	NT
N56	1	10/10	5/5 (4)
	2	0/10	–
	4	0/10	–
	5	0/10	–
	7	7/10	5/5 (4)
	8	0/10	–
	10	6/10	5/5 (4)
N97	1	0/10	–
	2	0/10	–
	3	0/10	–
	4	0/10	–
	5	0/10	–
	6	0/10	–
	7	0/10	–
	10	0/10	–

*Number of resistant plant/number of plants inoculated per line.
†The number of tested R_2 lines (derived from resistant R_1 plants) is indicated in parentheses.

(data not shown). As viral proteins were lacking, even in inoculated leaves, the resistant transgenic plants appeared to be immune to the virus. Progeny R_2 lines derived from resistant R_1 plants proved to be completely resistant to the virus (Table 1). In contrast to transgenic plant lines expressing the single-chain antibodies N3 (four of eight lines) and N56 (three of seven lines), none of the plants expressing scFv N97 was resistant to the virus, despite high levels of expression in several of the N97 lines (Fig. 2). Although considerably weaker than the recognition of TSWV, the N proteins of GRSV or TCSV are recognized by both N3 and N56 in ELISA (Griep *et al.*, 2000). However, additional challenging experiments of TSWV-resistant plant lines N3-1, N3-2, N56-1 and N56-7 with TCSV and GRSV revealed that the resistance was limited to TSWV (data not shown) and hence was insufficient to create a broad tospovirus resistance.

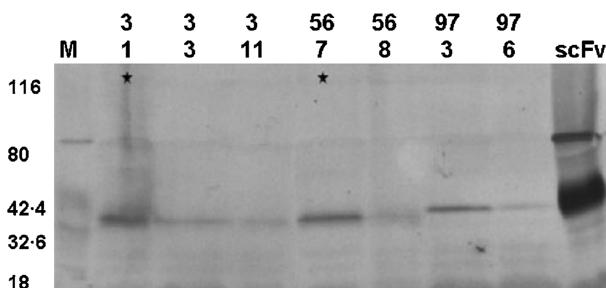

Fig. 2. Expression of anti-N scFvs in several lines (lower numbers) of the three different constructs (upper numbers). Sizes of the marker proteins (M) in kDa are indicated on the left. The scFv lane indicates the size of a single-chain antibody directed against a fungal cutinase produced in plants (Schouten *et al.*, 2002). The lines that displayed TSWV resistance are indicated with asterisks.

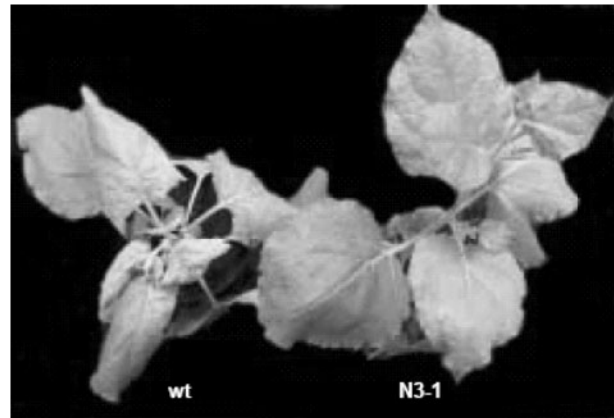

Fig. 3. Systemic symptom development of TSWV in wild-type *N. benthamiana* (wt) plants compared with transgenic plants expressing scFv N3-1. Plants were photographed at 10 days post-inoculation. In contrast to the TSWV-susceptible wt plant, the transgenic plant expressing the N3 scFv was completely resistant to the virus.

N3 and N56 block distinct early steps in virus replication

To substantiate further the observation that the phenotype of the resistant plants resembled immunity, we monitored virus replication in inoculated protoplasts as no viral proteins were found, even in the inoculated leaf. Protoplasts of several homozygous resistant R_2 lines were isolated and inoculated with TSWV. The accumulation of nucleoprotein over time was monitored by Western blot analysis. In protoplasts isolated from plants of lines N56-1 and N56-7, the amount of N protein after 2 days of incubation was lower than the input (Fig. 4); thus it could be concluded that these protoplasts completely failed to support TSWV replication. This showed that the observed resistance indeed operated at the single-cell level and most likely acted by inhibition of an early step in the virus replication cycle. Interestingly, the level of N protein in protoplasts derived from lines N3-1 and N3-2 (Fig. 4) remained constant, suggesting a limited amount of N protein production to

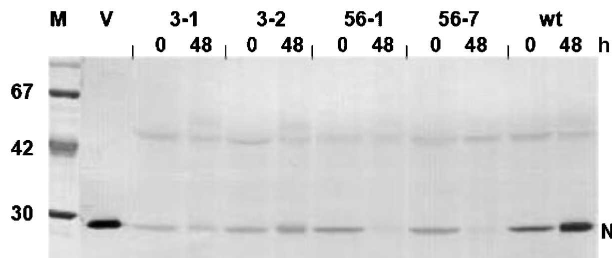

Fig. 4. Accumulation of TSWV N protein in virus-infected protoplasts of scFv-expressing resistant plants. M, protein size markers (kDa); V, purified virus used to inoculate the protoplasts. The resistant plant lines from which the protoplasts were isolated are indicated above the lanes, with the time of harvesting (h post-inoculation). The blot was incubated with a polyclonal antiserum against the viral N protein. The size of the nucleoprotein in indicated on the right (N).

compensate for degradation, although much less so than in wild-type protoplasts and insufficient for infection of the plant.

DISCUSSION

After the first report of successful transgenic plantibody-mediated delay of virus accumulation (Tavladoraki *et al.*, 1993), the number of reports on this subject of improving levels of resistance has remained limited until a recent successful study by Boonrod *et al.* (2004), which showed high levels of resistance using an RNA-dependent RNA polymerase-specific scFv. In recent years it has become clear that achieving high expression levels in plants of transgenic full-sized antibodies or derived fragments is more complicated than initially anticipated. Notably, the reducing environment of the plant's cytoplasm (Schouten *et al.*, 2002) has proved to be a difficult technical barrier to overcome, to obtain sufficient expression levels in this cell compartment where most plant viruses replicate. The increase in stability by the serendipitous addition of a KDEL sequence, originally added for ER retention of antibodies targeted to that compartment, proved a significant improvement (Schouten *et al.*, 1996, 1997). An alternative approach (de Jaeger *et al.*, 1999) demonstrated that phage display-derived scFvs possessed an intrinsically enhanced stability, possibly due to the phage-mediated selection procedure. In our previous research, we reported the generation of a panel of 12 different scFvs directed against the N protein of TSWV, as well as eight against the TSWV glycoproteins, targeting an assortment of epitopes (Griep *et al.*, 2000). The ability to generate a panel of target-binding antibodies is necessary to be able to select inhibiting antibody fragments. Using a selection of these scFvs directed against the viral N protein for expression in transgenic plants, it was demonstrated that combining phage display selection with the KDEL addition readily resulted in high levels of cytoplasmic expression of single-chain antibodies, although variation in transgenic protein levels was considerable. Besides the stability of the expressed protein, the expression of transgenes depends on the variability of the activity of the promoter due to the site of integration of the T-DNA, the insert copy number and the rate of gene silencing (de Neve *et al.*, 1999). Although not further investigated in this study, the factors mentioned may explain part of the observed variation in expression.

Resistance against TSWV was obtained in seven of the 23 lines, expressing either scFv N3 or N56 but not N97. Resistance was only observed in those transgenic plants that expressed high levels of scFv, but in addition depended on the significance of the interaction between the antibody and antigen. Even though lines expressing different scFvs had similar expression levels (e.g. N3-1, N56-7 and N97-3; Fig. 2), only the first two exhibited resistance. Apparently, binding of scFv N3 and N56 interfered with important functions of the N protein, such as replication of the virus, while binding of N97 did not. This showed that binding of an antibody to a viral protein, even when expressed at high levels, does not automatically imply effectiveness against the pathogen. It cannot be ruled out that the N97-targeted site in the N protein plays an important role in later phases of the infection cycle such as cell-to-cell transport or virus transmission, in which the N protein also plays a key role. During these phases, however, the high level of N protein produced by the replicating virus may saturate the expressed antibodies, thereby enabling the virus to escape their action. Protoplasts isolated from resistant plants of lines N56-1 and N56-7 completely failed to support TSWV replication and the production of N protein was completely blocked. As no N protein was produced at all in these plant cells (Fig. 4), this suggested that the very first step in the virus life cycle, viral transcription, may be blocked by the binding of the N56 scFv, for example by interfering with the cap-snatching process (Duijsings *et al.*, 2001). Plants expressing the N3 scFv were also highly resistant to the virus; however, at the single-cell level limited production of N protein was observed, albeit at levels much lower than in infected protoplasts of wild-type plants. The mode of operation of the N3 scFv may therefore be different from that of N56. Since a limited amount of N protein was produced in N3 plants, this could suggest that viral mRNAs are produced from inoculated viral RNAs, but that replication of the viral RNAs may be blocked. As no extra viral RNAs become available for transcription, the amount of mRNA remains low and only limited quantities of viral proteins are produced. These amounts are insufficient to complete the infection process and thus also lead to a virus-resistant phenotype on a whole-plant level. To increase the durability of resistance in practical applications, the two resistance-conferring antibodies N3 and N56 could be combined. As both antibodies are likely to target different epitopes, it will be more difficult for the virus to mutate in such a way that recognition by the two antibodies can be prevented.

In addition to the experiments using anti-N scFvs described

here, we also expressed several phage display-isolated single-chain antibodies targeting viral glycoproteins in an attempt to block virus transmission by thrips. Despite high expression of anti-G antibodies in the ER, where virus particles accumulate prior to uptake by thrips, at levels comparable to those of the N3 and N56 antibodies in resistant lines, no inhibition of virus transmission was observed (P. Maris and M. Prins, unpublished results). These findings indicate that the stoichiometry of the antigen–antibody interaction may play an important role in the successful application of plantibody-mediated resistance and it is therefore paramount that an early phase of the virus life cycle is targeted when amounts of viral proteins in the cell are still low.

As was shown here, antibodies can be targeted to the cytoplasm and produce effective virus resistance. Molecular tools are available to target these proteins to other parts of the plant cell, such as the ER, Golgi apparatus or apoplast. This broadens the scope of use of scFv proteins to other types of plant pathogens, such as fungi, bacteria and nematodes. Nematodes, for example, can be targeted by neutralizing essential excretion products (Popeijus *et al.*, 2000; Smant *et al.*, 1998) in either the cytoplasm or the extracellular space. As for viruses, using phage display-derived plantibodies to target proteins that are essential for early phases of the pathogenicity process could be crucial for successful applications in future resistance strategies.

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