Grapevine fanleaf virus (GFLV)-specific antibodies confer GFLV and *Arabidopsis mosaic virus* (ArMV) resistance in *Nicotiana benthamiana*

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SUMMARY

Grapevine fanleaf virus (GFLV) is one of the most destructive pathogens of grapevine. In this study, we generated monoclonal antibodies binding specifically to the coat protein of GFLV. Antibody FL₃, which bound most strongly to GFLV and showed cross-reactivity to *Arabidopsis mosaic virus* (ArMV), was used to construct the single-chain antibody fragment scFvGFLVcp-55. To evaluate the potential of this single-chain variable fragment (scFv) to confer antibody-mediated virus resistance, transgenic *Nicotiana benthamiana* plants were generated in which the scFv accumulated in the cytosol. Recombinant protein levels of up to 0.1% total soluble protein were achieved. The T₁ and T₂ progenies conferred partial or complete protection against GFLV on challenge with the viral pathogen. The resistance to GFLV in transgenic plants was strictly related to scFvGFLVcp-55 accumulation levels, confirming that the antibody fragment was functional *in planta* and responsible for the GFLV resistance. In addition, transgenic plants conferring complete protection to GFLV showed substantially enhanced tolerance to ArMV. We demonstrate the first step towards the control of grapevine fanleaf degeneration, as scFvGFLVcp-55 could be an ideal candidate for mediating nepovirus resistance.

INTRODUCTION

Fanleaf degeneration is a disease of grapevine in which new growth is dwarfed or deformed and leaves show characteristic vein banding or mottling, resulting in poor fruit quality and a reduced lifespan. It is caused predominantly by *Grapevine fanleaf virus* (GFLV), but also by *Arabidopsis mosaic virus* (ArMV), and is one of the most devastating and widespread viral diseases of grapevine, resulting in yield losses of up to 80% (Andret-Link

et al., 2004a; Martelli and Savino, 1991). GFLV belongs to subgroup A of the genus *Nepovirus* in the family *Comoviridae* (Mayo and Robinson, 1996). The virus is transmitted exclusively by *Xiphinema index* nematodes, which can survive in vineyard soils and retain GFLV for many years with or without host plants (Demangeat *et al.*, 2005). As no natural sources of resistance to this virus exist in *Vitis vinifera*, the control of fanleaf disease is based on sanitary selection and soil disinfection using nematicides. Although the dissemination of the virus has been reduced by these measures, the control of GFLV in naturally infected vineyards is still inefficient. The use of nematicides is largely unsuccessful because of the nematode's ability to exist on detached grape roots deep in the soil profile (Demangeat *et al.*, 2005; Raski and Goheen, 1988), and is forbidden in many countries because of environmental toxicity. Therefore, the development of virus-resistant grapevine varieties is likely to be the most effective, environmentally friendly and sustainable approach to grapevine fanleaf control.

Over the past 20 years, there has been enormous progress in virus resistance strategies based on genetic engineering. Transgenic plants have been created that express natural and engineered resistance genes, pathogen genes (pathogen-derived resistance) and antibodies against pathogen antigens (Nölke *et al.*, 2004). In grapevine, the most popular strategy has been the expression of viral coat protein (CP) genes, thus achieving CP-mediated resistance. Transgenic tobacco and grapevine plants expressing the CP of ArMV, *Grapevine leafroll associated virus-2* (GLRaV-2), GFLV, *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) have been reported (Gambino *et al.*, 2005; Gölles *et al.*, 1998, 2000; Gutonarov *et al.*, 2001; Krastanova *et al.*, 1995; Maghuly *et al.*, 2006; Martinelli *et al.*, 2000; Radian-Sade *et al.*, 2000; Spielmann *et al.*, 2000), and different levels of virus resistance have been reported in *N. benthamiana*. There have been few studies reporting enhanced resistance to GFLV achieved by targeting the GFLV CP. Bardonner *et al.* (1994) reported a significant delay in systemic GFLV infection in transgenic tobacco plants expressing GFLV CP, but no cross-protection was observed

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when transgenic plants were infected with ArMV. Transgenic grapevine plants expressing CP showed different symptoms, but no resistance to the disease (Barbier *et al.*, 1997). High levels of protection against GFLV have been reported by Monier *et al.* (2000) in transgenic *N. benthamiana* expressing nontranslatable CP sequences. Enhanced GFLV resistance was also observed after 3 years of field trials in 16% of transgenic grapevine lines producing GFLV CP, although there was no correlation between the expression level and degree of resistance (Vigne *et al.*, 2004).

In this study, we describe transgenic plants expressing an antibody that confers GFLV resistance. The antibody recognizes the GFLV CP, which was chosen as a target because it is the sole determinant of specific transmission by the nematode vector (Andret-Link *et al.*, 2004b), and is essential for systemic spreading of the virus in host plants (Belin *et al.*, 1999). We predicted that high-affinity antibodies specific to GFLV CP would neutralize the virus in the early stages of infection by interfering with genome encapsidation and preventing cell-to-cell movement. We demonstrate the engineering of a single-chain antibody fragment (scFvGFLVcp-55) that specifically binds to GFLV CP, and we report its effect on GFLV and ArMV infection *in planta*.

RESULTS

Production and characterization of GFLV-specific monoclonal antibodies

Mice were immunized with purified GFLV particles, and seven monoclonal antibodies (FL₁–FL₇) isolated from hybridoma clones were shown by enzyme-linked immunosorbent assay (ELISA) to bind purified GFLV particles *in vitro*. Five of the anti-GFLV antibodies belonged to the immunoglobulin G1 (IgG1) and κ isotype (FL₁, FL₃–FL₆), whereas FL₂ and FL₇ belonged to the IgG2a and κ isotype. FL₃ showed the highest reactivity for GFLV and also cross-reacted with ArMV particles (data not shown), and was therefore selected for further analysis.

Specific binding of FL₃ to the surface of GFLV particles was confirmed by immunosorbent electron microscopy. To reduce nonspecific binding, the GFLV particles were adsorbed on to grids coated with anti-GFLV polyclonal rabbit antibody before exposure to GFLV. Immunogold labelling revealed that FL₃ bound specifically to GFLV particles (Fig. 1A). No binding was observed in the negative control, which lacked FL₃ (Fig. 1B). This confirmed that the epitope recognized by FL₃ was located on GFLV CP and was accessible on the external surface of the virus capsid.

Isolation and characterization of a GFLV-specific murine single-chain variable fragment (scFv)

An scFv was engineered from cDNAs encoding the heavy and light chain variable regions of FL₃, and named scFvGFLVcp-55.

Fig. 1 Immunogold labelling of *Grapevine fanleaf virus* (GFLV) particles with monoclonal antibody FL₃. Purified GFLV particles were trapped on microscope grids coated with a polyclonal rabbit anti-GFLV antibody, washed and blocked with 0.1% (w/v) bovine serum albumin (BSA). The absorbed virus particles were subsequently incubated with 1 μ g/mL of affinity-purified FL₃ (A) or without FL₃ as a negative control (B). This was followed by the addition of goat-anti-mouse antibodies conjugated to 5-nm gold particles (1 : 50). Arrows indicate immunogold-labelled virus particles. Bar: 20 nm (A), 100 nm (B).

The scFv was produced in bacteria and purified by affinity chromatography. Specific binding to CP of GFLV isolated from infected *N. benthamiana* leaves was confirmed by ELISA, with no reaction to extracts from uninfected plants (Fig. 2). The scFv also recognized the closely related nepovirus ArMV and produced a signal of similar intensity to that seen with GFLV, confirming the cross-reactivity of the parental monoclonal antibody FL₃ and indicating the presence of a similar epitope on both viruses (Fig. 2).

Accumulation of scFvGFLVcp-55 in the cytosol

Because most of the GFLV infection cycle takes place in the plant cell cytosol, we placed the cDNA encoding scFvGFLVcp-55 into a plant expression cassette enabling cytosolic accumulation of the recombinant protein under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter (Fig. 3A). We carried out transient expression assays involving the vacuum infiltration of detached *N. benthamiana* leaves to facilitate the

Fig. 2 Reactivity of affinity-purified scFvGFLVcp-55 to *Grapevine fanleaf virus* (GFLV)- or *Arabidopsis mosaic virus* (ArMV)-infected *Nicotiana benthamiana* material. Polyclonal rabbit antibodies (1.5 µg/mL) specific to the GFLV coat protein were coated onto a microtitre plate and blocked with 200 µL of 5% (w/v) skimmed milk powder dissolved in 1 × PBS-T [1 × phosphate-buffered saline with 0.05% (v/v) Tween-20], before adding extract from GFLV- or ArMV-infected or noninfected *N. benthamiana* leaf material. Several concentrations of scFvGFLVcp-55 (25–600 ng) were applied. Bound scFv was detected with the 9E10 monoclonal antibody (0.3 µg/mL) and horseradish peroxidase-conjugated goat–anti-mouse (GAM^{HRP}) polyclonal antibody (0.16 µg/mL), followed by the addition of 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) substrate.

rapid and reliable evaluation of protein production and stability. Quantitative analysis against scFv standards produced in bacteria showed that scFvGFLVcp-55 accumulated to detectable levels in the cytosol of *N. benthamiana* leaves [approximately 2.45 µg/g fresh weight; 0.07% of total soluble protein (TSP)] (data not shown) and retained its specificity for GFLV as verified by ELISA (Fig. 3B). No cross-reaction to protein extracts from healthy *N. benthamiana* control leaves was observed.

Generation of transgenic plants and evaluation of virus resistance

To determine whether scFvGFLVcp-55 conferred virus resistance *in planta*, 22 transgenic *T₀* *N. benthamiana* plants were regenerated and screened for scFvGFLVcp-55 accumulation by ELISA (data not shown). Two *T₀* lines, #11 and #20, with the highest levels of scFvGFLVcp-55 (1.4 and 2.5 µg/g fresh weight; 0.04% and 0.07% of TSP) were used to establish the *T₁* generation. The accumulation of the recombinant protein in *T₁* plants was comparable with that in the parental *T₀* lines. To exclude somaclonal variation in plants derived directly from tissue culture (Kaeppeler *et al.*, 2000), scFvGFLVcp-55-mediated GFLV titre reduction was evaluated in both *T₁* and *T₂* plants. Fifteen *T₁* plants each from *T₀* lines #11 and #20 were inoculated with GFLV (20 µg/mL) and

Fig. 3 Functional analysis of cytosolic scFvGFLVcp-55 produced transiently in *Nicotiana benthamiana* leaves. (A) Plant expression cassette targeting scFvGFLVcp-55 to the plant cell cytosol. P, cauliflower mosaic virus (CaMV) 35S promoter with double enhancer; Ω, omega leader region of tobacco mosaic virus (TMV) RNA; Pw, 3' untranslated region of TMV RNA; c-myc, Myc epitope; His6, His₆ tag. (B) Binding analysis of transiently produced cytosolic scFvGFLVcp-55 to *Grapevine fanleaf virus* (GFLV)-infected or noninfected *N. benthamiana* material. Leaf extracts from GFLV-infected or noninfected leaves were added to a microtitre plate coated with a GFLV-specific polyclonal rabbit antibody (1.5 µg/mL), and total soluble protein from wild-type and transiently transformed *N. benthamiana* leaves was serially diluted in protein extraction buffer and added to the wells. Bound scFv fragments were detected with the 9E10 monoclonal antibody (0.3 µg/mL) and horseradish peroxidase-conjugated goat–anti-mouse (GAM^{HRP}) polyclonal antibody (0.16 µg/mL), followed by the addition of 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) substrate.

symptom development was monitored daily. Twenty wild-type *N. benthamiana* plants were assayed under the same conditions. At 14 days post-inoculation, virus accumulation in systemically infected leaves was analysed by ELISA. Seven *T₁* plants (#11-1, #11-10, #11-11 and #11-12; #20-1, #20-11 and #20-13), with antibody levels ranging from 0.17 to 3.5 µg/g fresh weight (corresponding to 0.005%–0.1% of TSP), conferred varying degrees of GFLV resistance (17%–100%) compared with systemically infected wild-type *N. benthamiana* plants. The remaining 23 transgenic *T₁* lines accumulated less than 0.005 µg/g fresh weight of scFvGFLVcp-55 and showed severe symptoms of GFLV systemic infection (data not shown). To confirm these results, virus challenge experiments were repeated on the *T₂* progeny of the seven self-fertilized *T₁* plants showing a significant reduction of GFLV titre. Fifteen *T₂* plants from each of the seven parental *T₁* lines and 20 nontransgenic control plants were inoculated with

Fig. 4 Evaluation of transgenic lines challenged with *Grapevine fanleaf virus* (GFLV). (A) Analysis of GFLV accumulation in wild-type and transgenic *T₂* *Nicotiana benthamiana* lines. Values are the means $\pm$ standard error (SE) for wild-type plants (WT) ($n = 20$) and transgenic lines ($n = 15$). WT, GFLV-infected wild-type *N. benthamiana*; #11-1, #11-10, #11-11, #11-12, #20-1, #20-11, #20-13, 105 transgenic *T₂* lines (15 plants per line) infected with GFLV. The reduction of GFLV accumulation compared with WT-infected plants is shown as a percentage. Leaf extracts from inoculated plants were incubated on enzyme-linked immunosorbent assay (ELISA) plates coated with anti-GFLV antibody (1.5 μ g/mL). Detection: anti-GFLV^{AP} antibody (1 : 1000). (B) Relationship between scFvGFLVcp-55 accumulation and reduction of GFLV titre in transgenic *T₂* lines. FW, fresh weight.

GFLV. All 105 challenged transgenic plants showed enhanced or complete GFLV resistance, ranging from 27% to 100%, compared with infected control plants (Fig. 4A). No virus could be detected by ELISA in the upper leaves of any of the 15 *T₂* progenies generated from the parental lines #11-10, #11-11 and #20-11, demonstrating that these plants were completely resistant to GFLV infection. The remaining 60 *T₂* plants from the parental lines #11-1, #11-12, #20-1 and #20-13 showed 27%–84% enhanced resistance to GFLV compared with infected control plants. Resistant plants showed no evidence of systemic virus infection, even at 60 days post-inoculation. Levels of virus titre reduction correlated strictly with the level of scFv accumulation. Antibody levels greater than 1.8 μ g/g led to a complete absence of the

Fig. 5 Evaluation of transgenic lines challenged with *Arabis mosaic virus* (ArMV). (A) Analysis of ArMV cross-resistance in wild-type and transgenic *T₂* *Nicotiana benthamiana* lines. Values are means $\pm$ standard error (SE) for wild-type plants (WT) ($n = 20$) and transgenic lines ($n = 15$). WT, ArMV-infected wild-type *N. benthamiana*; #11-1, #11-10, #11-11, #11-12, #20-1, #20-11, #20-13, 105 transgenic *T₂* lines (15 plants per line) infected with ArMV. The change in ArMV accumulation compared with WT-infected plants is shown as a percentage. Leaf extracts from inoculated plants were incubated on enzyme-linked immunosorbent assay (ELISA) plates coated with anti-ArMV antibody (1.5 μ g/mL). Detection: anti-ArMV^{AP} antibody (1 : 1000). (B) Relationship between scFvGFLVcp-55 accumulation and ArMV cross-tolerance in transgenic *T₂* lines. FW, fresh weight.

virus, whereas lower scFv levels resulted in proportionately reduced GFLV titres on inoculation. The correlation between antibody accumulation and resistance yielded a high correlation coefficient (Fig. 4B).

To evaluate the effect of scFvGFLVcp-55 on ArMV infection, 105 *T₂* lines showing a complete absence of the virus in systemic leaves (#11-10, #11-11, #20-11) or significantly reduced GFLV titres (#11-1, #11-12, #20-1, #20-13) were infected with ArMV particles. There was a significant level of ArMV titre reduction (19%–87%) in all the transgenic lines (Fig. 5A). Again, higher antibody levels resulted in improved resistance, and the correlation between scFvGFLVcp-55 levels and ArMV titre reduction

confirmed that scFvGFLVcp-55 expression is an appropriate strategy to confer improved ArMV resistance (Fig. 5B).

DISCUSSION

The goal of this study was to create transgenic *N. benthamiana* plants showing antibody-mediated resistance to GFLV, an agent responsible for one of the most devastating diseases of commercial grapevine. The use of antibodies to confer resistance against plant pathogens has been reported previously (Boonrod *et al.*, 2004; Gargouri-Bouazid *et al.*, 2006; Prins *et al.*, 2005; Villani *et al.*, 2005), and the greatest success has been achieved when antibodies against viruses are expressed in the cytosol, where the viral pathogen is most vulnerable. As antibody heavy and light chains do not always assemble into a functional protein in the reducing environment of the cytosol (Schillberg *et al.*, 1999), scFv antibody fragments are preferable to full-sized Igs because they are small and do not need to assemble from separate subunits. Despite these advantages, however, even scFv antibodies can find the cytosol inhospitable, and an enormous range of production levels have been reported from poor or undetectable (for a review, see Nölke *et al.*, 2004) to very high, indicating that certain scaffold sequences may promote scFv stability and functionality (Nölke *et al.*, 2005; Prins *et al.*, 2005; Tavladoraki *et al.*, 1999; Villani *et al.*, 2005). In this study, scFvGFLVcp-55 accumulated to moderate levels (0.002%–0.1% TSP) in the cytosol, but this was sufficient to confer GFLV resistance and improved ArMV tolerance in transgenic plants. Importantly, the antigen-binding capacity of scFvGFLVcp-55 was retained in the cytosol, although we cannot rule out the possibility that binding was less efficient *in planta* compared with the nonreducing conditions in ELISA, where disulphide bonds form spontaneously. Nevertheless, it has been demonstrated that some scFv antibodies can tolerate the reducing conditions in the plant cell cytosol (Tavladoraki *et al.*, 1999) and even form intra-disulphide bonds in this environment (Schouten *et al.*, 2002).

Interestingly, the parental monoclonal antibody FL₃ and its scFv derivative bound to GFLV and ArMV particles, indicating that a similar epitope is present on the CP of both viruses, which are closely related nepoviruses. Their CPs share 69% identity at the primary sequence level (Steinkellner *et al.*, 1992), and the cross-reaction of ArMV-specific monoclonal antibodies with GFLV has been reported previously (Frison and Stace-Smith, 1992). Studies to define the putative binding epitope of FL₃ and scFvGFLVcp-55 are in progress.

GFLV particles assemble from multiple copies of a multifunctional CP that plays an important role in nematode transmission, virus encapsidation, and cell-to-cell and systemic movement in plants (Andret-Link *et al.*, 2004b; Belin *et al.*, 1999; Callaway *et al.*, 2001). CP appeared to be an ideal target antigen for antibody-mediated resistance to GFLV and, in confirmation, we observed that constitutive expression of CP-specific scFvGFLVcp-55

in the cytosol conferred resistance to GFLV in transgenic *N. benthamiana* plants. All 105 T₂ progeny of self-fertilized T₁ plants expressing the antibody showed significantly reduced virus titre or complete GFLV resistance in response to virus challenge. The transgenic lines also showed enhanced tolerance to ArMV, making this scFv valuable for engineering broad resistance to related nepoviruses.

GFLV spreads from cell to cell through tubular structures generated by the virus-encoded movement protein (Ritzenthaler *et al.*, 1995a; Ritzenthaler *et al.*, 1995b). Although limited information is available on this process, it has been suggested that specific interaction between the nine C-terminal residues of the movement protein and CP are crucial for systemic spread (Belin *et al.*, 1999). In a previous study, Andret-Link *et al.* (2004b) highlighted that the structure or charge (rather than the sequence) of the nine C-terminal residues of the GFLV movement protein is critical for effective interaction with CP. As resistant transgenic plants showed no evidence of systemic spread of the virus, we suggest that scFvGFLVcp-55 binds to CP in such a way as to block the specific interaction with the movement protein through direct competition or by interfering with CP folding, so that interaction is no longer possible. However, further studies need to be performed to support this hypothesis.

On the basis of our data, we propose that scFvGFLVcp-55 can achieve complete GFLV resistance following mechanical inoculation when present at levels above 0.05% TSP, a smaller amount than that previously reported for anti-CP scFvs against *Artichoke mottle crinkle virus* and *Cucumber mosaic virus* (Tavladoraki *et al.*, 1993; Villani *et al.*, 2005) and for an anti-nucleoprotein scFv against *Tomato spotted wilt virus* (Prins *et al.*, 2005). In contrast, complete resistance against *Tomato bushy stunt virus* in *N. benthamiana* was achieved by expressing an scFv against viral RNA-dependent RNA polymerase at 0.016% TSP (Boonrod *et al.*, 2004). A correlation between scFv levels and the degree of virus resistance has been observed in other studies of antibody-mediated resistance (Boonrod *et al.*, 2004; Schillberg *et al.*, 2000; Villani *et al.*, 2005).

Constitutive expression of a GFLV-specific scFv in the plant cytosol resulted in the complete absence of GFLV and significantly reduced ArMV titres in *N. benthamiana*. As mechanical inoculation represents a much greater challenge than natural, nematode-borne infections, it is probable that antibody-mediated control of GFLV and ArMV would protect transgenic plants under normal field conditions. We are currently performing experiments to determine whether transgenic grapevine plants expressing scFvGFLVcp-55 can be used to engineer broad-range resistance to nepoviruses. In conclusion, we have shown that antibody-mediated resistance can be used to confer nepovirus resistance in the model plant *N. benthamiana*, and may provide a powerful tool to create virus-resistant varieties of agriculturally important crops, such as grapevine.

EXPERIMENTAL PROCEDURES

Plasmid DNA, bacteria and plants

Plasmids pTRA (Sack *et al.*, 2007) and pHENHi (Peschen *et al.*, 2004) were used for recombinant protein expression in bacteria and plants, respectively. *Escherichia coli* strains DH5 α and HB2151 (Stratagene, Heidelberg, Germany) were used for general cloning and bacterial expression of recombinant scFvs, and *Agrobacterium tumefaciens* strain GV3101 (pMP90RK, Gm^R, Km^R, Rif^R) (Konz and Schell, 1986) was used for plant transformation. *N. benthamiana* plants were cultivated in the glasshouse in DE73 standard soil with a 16-h natural daylight photoperiod and a 25 °C/22 °C day/night temperature regime.

Propagation and purification of GFLV and ArMV

GFLV and ArMV isolates from infected Huxel and Pinot gris grapevines of Neustadt Weinstraße, respectively, were propagated on *Chenopodium quinoa*. The virus was purified as described by Pinck *et al.* (1988).

Monoclonal antibody production

BALB/c mice were immunized subcutaneously with 100 μ g of purified GFLV particles in 100 μ L of 1 $\times$ phosphate-buffered saline (PBS) emulsified with 40 μ L of GERBU's adjuvant (GERBU Biochemicals GmbH, Gaiberg, Germany). Two booster injections of the same material were given 2 and 4 weeks later. Thirty days later, mice received the last intraperitoneal boost of GFLV (50 μ g). Splenocytes were collected 3 days later and immortalized by fusion with mouse myeloma cells (SP2/0-Ag 8) (Köhler and Milstein, 1975). Cells grown in 24-well plates were screened for binding to GFLV particles by capture ELISA (see below). Hybridomas from positive cells were cloned by limiting dilution and plated on microtitre plates, and the secretion of mouse monoclonal antibodies specific to GFLV was confirmed by capture ELISA. Monoclonal antibodies were purified from the hybridoma supernatant via Protein A chromatography, as recommended by the manufacturer (Invitrogen, Karlsruhe, Germany). The isotype of the affinity-purified murine monoclonal antibodies was identified by ELISA using the isotyping ELISA kit, according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA).

Cloning the scFv coding sequence

Total RNA was extracted from 2.5×10^7 freshly grown hybridoma cells secreting the anti-GFLV CP antibody using Trizol reagent (Invitrogen). First-strand cDNA was prepared using the SuperscriptTM Preamplification System Kit (Invitrogen) with the following

specific primers: murine IgG1 heavy chain (5'-TAACCCTWGAC-CAGGCATCC-3') and murine κ light chain (5'-GCTGATGCTGCAC-CAATGTATCCGTCGACGCGCGCGACTAGT-3'). Mouse antibody heavy and light chain variable regions were amplified by PCR using a set of degenerate primers binding to the first and fourth framework regions of the murine IgG1 heavy chain and κ light chain, as described by Nölke *et al.* (2005). The PCR products were gel purified and introduced into the bacterial expression vector pHENHi, containing a modified 218-linker (Whitlow *et al.*, 1993) and C-terminal c-myc and His₆ epitope tags. Individual clones were tested for binding specificity by ELISA against control antigens, using soluble scFv fragments produced in *E. coli* HB2151.

Expression of scFv in *E. coli* and affinity purification

A single colony of recombinant *E. coli* strain HB2151 was transferred to 2 $\times$ TY (10 g tryptone, 1 g yeast, 0.5 g NaCl) medium containing 100 μ g/mL ampicillin and 1% (w/v) glucose, and incubated overnight at 37 °C with shaking (200 r.p.m.). Two millilitres of overnight culture were transferred to 500 mL of 2 $\times$ TY-ampicillin medium containing 0.1% (w/v) glucose, and grown to an optical density at 600 nm (OD₆₀₀) of 0.7–0.9. After the addition of β -D-isopropyl-thiogalactopyranoside to a final concentration of 1 mM, the culture was incubated at 28 °C overnight. Soluble scFv antibody fragments were affinity purified by immobilized metal affinity chromatography (IMAC; Qiagen, Hilden, Germany).

ELISA

Microtitre plates (high-binding; Greiner Bio-One GmbH, Frickenhausen, Germany) were coated overnight at 4 °C with polyclonal rabbit anti-GFLV (1.5 μ g/mL) or anti-ArMV (1.5 μ g/mL) (Bioreba, Reinach BL1, Switzerland) IgGs in 50 mM carbonate-bicarbonate buffer (pH 9.6). Wells were blocked with 200 μ L of 5% (w/v) skimmed milk dissolved in 1 $\times$ PBS with 0.05% (v/v) Tween-20 (1 $\times$ PBS-T) for 1 h at room temperature, and then incubated with 100 μ L of TSP extracts from *N. benthamiana* material, either infected or not infected with GFLV (Bioreba). Finally, serial concentrations (25–600 ng or 40–2560 ng) of IMAC-purified scFv produced in *E. coli* or transiently expressed in *N. benthamiana* were added to the extracts, and incubated for 2 h at room temperature. Bound scFvs were revealed by the addition of the anti-c-myc 9E10 monoclonal antibody (0.3 μ g/mL) (ATCC number CRL-1729) and a goat-anti-mouse secondary antibody conjugated to horseradish peroxidase (GAM^{HRP}; 0.16 μ g/mL) (Jackson ImmunoResearch Laboratories) in PBS-T with 1% (w/v) skimmed milk powder. Between each step, the plates were washed with PBS-T. ELISA readings (OD_{405 nm}) were taken after 1 h of incubation with 100 μ L of 1 mg/mL 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) substrate in 70 mM citrate phosphate buffer, pH 4.2, containing a 1 : 1000 dilution of 30% H₂O₂ at 37 °C.

Electron microscopy

Electron microscope grids with a Pioloform-carbon support film were floated for 5 min on drops containing 1.5 µg/mL of polyclonal rabbit anti-GFLV IgG particles (Bioreba) in phosphate buffer (1 × PBS, pH 7.2). The grids were washed with 1 × PBS-T and then blocked with 0.1% (w/v) bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in 1 × PBS. The purified GFLV particles (0.01 µg in 100 µL of 1 × PBS) were then absorbed on to the grids for 1 h at room temperature, followed by incubation with the affinity-purified monoclonal antibody FL₃ (1 µg/mL) for 90 min at room temperature. After washing with 1 × PBS-T, samples were decorated with 1.5 µg/mL of gold-labelled goat-anti-mouse IgG (5-nm gold particles; British Biocell, UK) in 1 × PBS-T for 2 h at room temperature. Finally, the grids were extensively washed, followed by staining with five drops of 2% (w/v) uranyl acetate. Finally, a Philips EM 400T electron microscope was used (Institute of Neuropathology, University Hospital, RWTH Aachen University, Germany) to analyse the monoclonal antibody binding to viral particles.

Construction of the plant expression cassettes

The scFvGFLVcp-55 cDNA was inserted into a pUC18 derivative (Yanisch-Perron *et al.*, 1985) using the *Nco*I and *Sal*I restriction sites. The pUC18-derived plasmid contained the tobacco mosaic virus (TMV) 5' untranslated region (Ω leader) (Schmitz *et al.*, 1996) and the c-Myc and His₆ tags. The complete cassette was then excised using *Eco*RI and *Xba*I, and inserted into the plant expression vector pTRA (Sack *et al.*, 2007) between the double-enhanced 35S promoter (Kay *et al.*, 1987) and the TMV 3' untranslated region (Pw). The final construct was named pTRA-35SS:scFvGFLVcp-55.

Transformation of *N. benthamiana*

The plant expression vectors were introduced into *A. tumefaciens* GV3101 cells using a Gene Pulser II electroporation system (BioRad, Hercules, CA, USA), according to the manufacturer's instructions. Recombinant protein accumulation in *N. benthamiana* leaves was evaluated using the vacuum-assisted transient expression assay described by Kapila *et al.* (1996) and Vaquero *et al.* (1999). Stably transformed *N. benthamiana* plants were generated by leaf disc transformation with recombinant *A. tumefaciens* (Horsch *et al.*, 1985). For the selection of bialaphos-resistant T₁ and/or T₂ plants, seeds from scFvGFLVcp-55-producing T₀ and/or T₁ plants were collected and germinated on Murashige and Skoog agar medium including vitamins (Duchefa, Haarlem, the Netherlands) supplemented with 2% (w/v) sucrose, 0.4 µg/mL thiamine and 5 µg/mL bialaphos.

Protein extraction and analysis

Plant leaves were ground to a fine powder under liquid nitrogen, and TSP was extracted with two volumes of extraction buffer [50 mM Tris-HCl, pH 8, 100 mM NaCl, 10 mM dithiothreitol (DTT), 5 mM ethylenediaminetetraacetic acid (EDTA) and 0.1% (v/v) Tween-20]. The extracts were centrifuged at 8500 *g* for 20 min at 4 °C. The concentration of extracted soluble protein was determined in triplicate by the BioRad Protein Assay (BioRad), with bovine serum albumin as a standard. The amount of scFvGFLVcp-55 was quantified by ELISA using known concentrations of affinity-purified scFvGFLVcp-55 as a standard. The scFvs were detected with an anti-c-myc (9E10) monoclonal antibody (0.3 µg/mL) and a horseradish peroxidase-conjugated goat-anti-mouse secondary antibody (GAM^{HRP}; 0.16 µg/mL) (Jackson ImmunoResearch Laboratories).

Virus challenge of transgenic *N. benthamiana* plants

GFLV and ArMV virus challenge was performed with wild-type as well as T₁ and T₂ generations of self-pollinated transgenic *N. benthamiana* plants selected on bialaphos. Three upper leaves of wild-type and transgenic plants, at the five-leaf stage, were inoculated mechanically by rubbing with 50 µL of virus solution (20 µg/mL) consisting of extracts from young symptomatic leaves of *N. benthamiana* infected with GFLV and ArMV, ground into two volumes of freshly prepared ice-cold bentonite buffer. The amount of virus used for challenge inoculation was quantified by direct ELISA using known concentrations of purified GFLV and ArMV as standard. Viruses were detected with anti-GFLV or anti-ArMV monoclonal antibody conjugated with alkaline phosphatase diluted 1 : 1000 in 1 × PBS (Bioreba). The infected plants were maintained in a glasshouse at 25 °C for up to 9 weeks post-inoculation.

Virus accumulation in ArMV- and GFLV-inoculated and systemically infected leaves was determined by a double-sandwich ELISA using ArMV and GFLV detection kits, according to the manufacturer's instructions (Bioreba). Leaf samples were taken from each of the three inoculated leaves to verify the inoculation event, and three upper leaves from about the same position on each plant were taken to determine systemic infection.

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REFERENCES

- Andret-Link, P., Laporte, C., Valat, L., Ritzenthaler, C., Demangeat, G., Vigne, E., Laval, V., Pfeiffer, P., Stussi-Garaud, C. and Fuchs, M. (2004a) Grapevine fanleaf virus: still a major threat to the grapevine industry. *J. Plant Pathol.* **86**, 183–195.

- Andret-Link, P., Schmitt-Keichinger, C., Demangeat, G., Komar, V. and Fuchs, M. (2004b) The specific transmission of *Grapevine fanleaf virus* by its nematode vector *Xiphinema index* is solely determined by the viral coat protein. *Virology*, **320**, 12–22.
- Barbier, P., Demangeat, G., Perrin, M., Cobanov, P., Jacquet, C. and Walter, B. (1997) Grapevine genetically transformed with the coat protein gene of grapevine fanleaf virus: an analysis of transformants. In: *Extended Abstracts of the 12th ICVG Meeting, Lisbon, Portugal, 28 September–2 October 1997* (Sequera, O., Sequera, J. and Santos, M., eds), p. 131. Lisbon.
- Bardonnier, N., Hans, F., Serghini, M.A. and Pinck, L. (1994) Protection against virus infection in tobacco plants expressing the coat protein of grapevine fanleaf nepovirus. *Plant Cell Rep.* **13**, 357–360.
- Belin, C., Schmitt, C., Gaire, F., Walter, B., Demangeat, G. and Pinck, L. (1999) The nine C-terminal residues of the grapevine fanleaf nepovirus movement protein are critical for systemic virus spread. *J. Gen. Virol.* **80**, 1347–1356.
- Boonrod, K., Galetzka, D., Nagy, P.D., Conrad, U. and Krczal, G. (2004) Single-chain antibodies against a plant viral RNA-dependent RNA polymerase confer virus resistance. *Nat. Biotechnol.* **22**, 856–862.
- Callaway, A., Giesman-Cookmeyer, D., Gillock, E.T., Sit, T.L. and Lommel, S.A. (2001) The multifunctional capsid protein of plant viruses. *Annu. Rev. Phytopathol.* **39**, 419–460.
- Demangeat, G., Voisini, R., Minot, J.C., Bosselut, N., Fuchs, M. and Esmenjaud, D. (2005) Survival of *Xiphinema index* in vineyard soil and retentions of grapevine fanleaf virus over extended time in the absence of host plants. *Phytopathology*, **95**, 1151–1156.
- Frison, E.A. and Stace-Smith, R. (1992) Cross-reacting and heterospecific monoclonal antibodies produced against arabis mosaic nepovirus. *J. Gen. Virol.* **73**, 2525–2530.
- Gambino, G., Gribaudo, I., Leopold, S., Scharl, A. and Laimer, M. (2005) Molecular characterization of grapevine plants transformed with GFLV resistance genes: I. *Plant Cell Rep.* **24**, 655–662.
- Gargouri-Bouazid, R., Jaouna, L., Rouis, S., Saidi, M.N., Bouaziz, D. and Ellouz, R. (2006) PVY-resistant transgenic potato plants expressing an anti-Nla protein scFv antibody. *Mol. Biotechnol.* **33**, 133–140.
- Gölles, R., da Câmara Machado, A., Thoubert, A., Moser, R., Kantiger, H. and Laimer da Câmara Machado, M. (1998) Transformation of somatic embryos of *Vitis* sp. with different constructs containing nucleotide sequences from nepovirus coat protein genes. *Acta Hort.* **447**, 265–270.
- Gölles, R., Moser, R., Puhrieger, H., Kantiger, H., da Câmara Machado, M.L., Minafra, A., Savino, V., Saldarelli, P., Martelli, G.P. and da Câmara Machado, A. (2000) Transgenic grapevine expressing coat protein gene sequences of grapevine fanleaf virus, arabis mosaic virus, grapevine virus A and grapevine virus B. *Acta Hort.* **528**, 305–311.
- Gutonarov, G.P., Tsvetkov, I.J., Colova-Tsolova, V.M. and Atanassov, A.I. (2001) Genetically engineered grapevines carrying GFLV coat protein and antifreeze genes. *Agric. Conspec. Sci.* **66**, 69–74.
- Horsch, R.B., Fry, J.E., Hoffman, N.L., Rogers, S.G. and Fraley, R.T. (1985) A simple and general method for transferring genes into plants. *Science*, **227**, 1229–1231.
- Kaeppler, S.M., Kaeppler, H.F. and Rhee, Y. (2000) Epigenetic aspects of somaclonal variation in plants. *Plant Mol. Biol.* **43**, 179–188.
- Kapila, J., De Rycke, R., van Montagu, M. and Angenon, G. (1996) An *Agrobacterium* mediated transient gene expression system for intact leaves. *Plant Sci.* **122**, 101–108.
- Kay, R., Chan, A. and McPherson, J. (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science*, **236**, 1299–1302.
- Köhler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibodies of preferred specificity. *Nature*, **256**, 495–497.
- Konz, C.B. and Schell, J. (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol. Gen. Genet.* **204**, 382–396.
- Krastanova, S., Perrin, M., Barbier, P., Demangeat, P., Cornuet, P., Bardonnet, N., Otten, L., Pinck, L. and Walter, B. (1995) Transformation of grapevine rootstocks with the coat protein gene of grapevine fanleaf nepovirus. *Plant Cell Rep.* **14**, 550–554.
- Maghuly, F., Leopold, S., da Câmara Machado, A., Fernandez, E.B., Khan, M.A., Gambino, G., Gribaudo, I., Scharl, A. and Laimer, M. (2006) Molecular characterization of grapevine plants transformed with GFLV resistance genes: II. *Plant Cell Rep.* **25**, 546–553.
- Martelli, G.P. and Savino, V. (1991) Fanleaf degeneration. In: *Compendium of Grape Diseases* (Pearson, R.C. and Goheen, A., eds), pp. 48–49. St. Paul, MN: APS Press.
- Martinelli, L., Costa, D., Poletti, V., Festi, S., Buzkan, N., Minafra, A., Saldarelli, P., Martelli, G.P. and Perl, A. (2000) Genetic transformation of tobacco and grapevine for resistance to viruses related to rugose wood disease complex. *Acta Hort.* **528**, 321–327.
- Mayo, M.A. and Robinson, D.J. (1996) Nepoviruses: molecular biology and replication. In: *The Plant Viruses, Polyhedral Virions and Bipartite RNA*, Vol. 5 (Harrison, B.D. and Murrant, A.F., eds), pp. 139–185. New York: Plenum.
- Monier, C., Barbier, P. and Walter, B. (2000) Protection against grapevine fanleaf virus in transgenic tobacco containing non-translatable sequences. *Acta Hort.* **528**, 379–383.
- Nölke, G., Fischer, R. and Schillberg, S. (2004) Antibody-based pathogen resistance in plants. *J. Plant Pathol.* **86**, 5–17.
- Nölke, G., Schneider, B., Fischer, R. and Schillberg, S. (2005) Immunomodulation of polyamine biosynthesis in tobacco plants has a significant impact on polyamine levels and generates a dwarf phenotype. *Plant Biotechnol. J.* **3**, 237–247.
- Peschen, D., Li, H., Fischer, R., Kreuzaler, F. and Liao, Y. (2004) Fusion proteins comprising a *Fusarium*-specific antibody linked to an antifungal peptide protect plants against a fungal pathogen. *Nat. Biotechnol.* **22**, 732–738.
- Pinck, L., Fuchs, M., Pinck, M., Ravelonandro, M. and Walter, B. (1988) A satellite RNA in grapevine fanleaf virus strain F13. *J. Gen. Virol.* **69**, 233–239.
- Prins, M., Lohuis, D., Schots, A. and Goldbach, R. (2005) Phage display-selected single-chain antibodies confer high levels of resistance against *Tomato spotted wilt virus*. *J. Gen. Virol.* **86**, 2107–2113.
- Radian-Sade, S., Perl, A., Edelbaum, O., Kuznetsova, L., Gafny, R., Sela, I. and Tanne, E. (2000) Transgenic *Nicotiana benthamiana* and grapevine plant transformed with grapevine virus A (GVA) sequences. *Phytoparasitica*, **28**, 79–86.
- Raski, D.J. and Goheen, A.C. (1988) Comparison of 1,3-dichloropropene and methyl bromide for control of *Xiphinema index* and grapevine fanleaf degeneration complex. *Am. J. Enol. Vitic.* **39**, 334–336.
- Ritzenthaler, C., Pinck, M. and Pinck, L. (1995a) Grapevine fanleaf nepovirus P38 putative movement protein is not transiently expressed and is a stable final maturation product in vivo. *J. Gen. Virol.* **76**, 907–915.
- Ritzenthaler, C., Schmit, A.C., Michler, P., Stussi-Graud, C. and Pinck, L. (1995b) Grapevine fanleaf nepovirus P38 putative movement

- protein is located on tubules *in vivo*. *Mol. Plant-Microbe Interact.* **8**, 379–387.
- Sack, M., Paetz, A., Kunert, R., Bomble, M., Hesse, F., Stiegler, G., Fischer, R., Katinger, H., Stoeger, E. and Rademacher, T. (2007) Functional analysis of the broadly neutralizing human anti-HIV-1 antibody 2F5 produced in transgenic BY-2 suspension cultures. *FASEB J.* **21**, 1655–1664.
- Schillberg, S., Zimmermann, S., Voss, A. and Fischer, R. (1999) Apoplastic and cytosolic expression of full-size antibodies and antibody fragments in *Nicotiana tabacum*. *Transgenic Res.* **8**, 255–263.
- Schillberg, S., Zimmermann, S., Findlay, K. and Fischer, R. (2000) Plasma membrane display of anti-viral single chain Fv fragments confers resistance to tobacco mosaic virus. *Mol. Breed.* **6**, 317–326.
- Schmitz, J., Prüfer, D., Rohde, W. and Tacke, E. (1996) Non-canonical translation mechanisms in plants: efficient *in vitro* and *in planta* initiation at AUU codons of the tobacco mosaic virus enhancer sequence. *Nucleic Acids Res.* **24**, 257–263.
- Schouten, A., Roosien, J., Bakker, J. and Schots, A. (2002) Formation of disulfide bridges by a single-chain Fv antibody in the reducing ectopic environment of the plant cytosol. *J. Biol. Chem.* **277**, 19 339–19 345.
- Spielmann, A., Krastanova, S., Douet-Orhant, V. and Gugerli, P. (2000). Analysis of transgenic grapevine (*Vitis rupestris*) and *Nicotiana benthamiana* plants expressing an Arabis mosaic virus coat protein gene. *Plant Sci.* **156**, 235–244.
- Steinkellner, H., Himmler, G., Sagl, R., Mattanovich, D. and Katinger, H. (1992) Amino-acid sequence comparison of nepovirus coat proteins. *Virus Genes*, **6**, 197–202.
- Tavladoraki, P., Benvenuto, E., Trinca, S., De Martins, D., Cattaneo, A. and Galeffi, P. (1993) Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. *Nature*, **366**, 469–472.
- Tavladoraki, P., Girotti, A., Donini, M., Arias, F.J., Mancini, C., Morea, V., Chiaraluce, R., Consalvi, V. and Benvenuto, E. (1999) A single-chain antibody fragment is functionally expressed in the cytoplasm of both *Escherichia coli* and transgenic plants. *Eur. J. Biochem.* **262**, 617–624.
- Vaquero, C., Sack, M., Chandler, J., Drossard, J., Schuster, F., Moncke, M., Schillberg, S. and Fischer, R. (1999) Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. *Proc. Natl. Acad. Sci. USA*, **96**, 11 128–11 133.
- Vigne, E., Komar, V. and Fuchs, M. (2004) Field safety assessment of recombination in transgenic grapevines expressing the coat protein gene of Grapevine fanleaf virus. *Transgenic Res.* **13**, 165–179.
- Villani, M.E., Roggero, P., Bitti, O., Benvenuto, E. and Franconi, R. (2005) Immunomodulation of cucumber mosaic virus infection by intrabodies selected *in vitro* from a stable single-framework phage display library. *Plant Mol. Biol.* **58**, 305–316.
- Whitlow, M., Bell, B.A., Feng, S.L., Filpula, D., Hardman, K.D., Hubert, S.L., Rollence, M.L., Wood, J.F., Schott, M.E. and Milenic, D.E. (1993) An improved linker for single chain Fv with reduced aggregation and enhanced proteolytic stability. *Protein Eng.* **6**, 989–995.
- Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*, **33**, 103–119.