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ABSTRACT

A single-chain variable antibody fragment (scFv) library tested against the non-structural NSP5 protein of human rotavirus A was screened by a yeast two-hybrid system against three proteins derived from the RNA-dependent RNA polymerase (RdRp) of cucumber mosaic virus (CMV), with the aim of blocking their function and preventing viral infection once expressed *in planta*. The constructs tested were (i) '2a' consisting of the full-length 2a gene (839 amino acids, aa), (ii) 'Motifs' covering the conserved RdRp motifs (IV-VII) (132 aa) and (iii) 'GDD' located within the conserved RdRp motif VI (GDD, 22 aa). The '2a' and 'Motifs' constructs interacted with 96 and 25 library constructs, respectively, while the 'GDD' construct caused transactivation. The scFvs positive in yeast two-hybrid system were analyzed *in vivo* for their interaction with the 2a and Motifs proteins in a mammalian transient expression system. Eighteen tobacco lines stably transformed with four selected scFvs were produced and screened for resistance against two different CMV isolates. Different levels of resistance and rate of recovery were observed with CMV of both groups I and II, particularly in lines expressing intrabodies against the full-length 2a protein. This work describes for the first time the use of intrabodies against the RdRp of CMV to obtain plants that reduce infection of a pandemic virus, showing that the selected scFvs can modulate virus infection and induce premature recovery in tobacco plants.

Keywords: *Nicotiana tabacum; Solanaceae*; tobacco; *Cucumber mosaic virus* (CMV); yeast two-hybrid system; transgenic plants; virus resistance; intrabodies; scFv; RNA-dependent RNA polymerase (RdRp).

1. Introduction

Cucumber mosaic virus (CMV) is the type species of the genus *Cucumovirus*, family *Bromoviridae* (Roossinck, 1999). It is an icosahedral virus with an approximate diameter of 30 nm. The CMV genome consists of three single-stranded genomic RNAs (RNAs 1, 2, and 3). RNAs 1 and 2 encode the 1a and 2a proteins, respectively (Jacquemond, 2012), which together form the viral replicase, part of the putative replication complex shown to localize to the tonoplast (Hayes and Buck, 1990; O'Reilly et al., 1998; Cillo et al., 2002). The 2a protein is the RNA-dependent RNA polymerase (RdRp). RdRps have highly conserved amino acid motifs (Koonin, 1991; O'Reilly and Kao, 1998) and a structure conserved even among distantly related viruses, consisting of "palm, thumb and finger" structural domains with four common amino acid motifs (A-D), including the GDD sequence in motif C, for magnesium co-ordination. The 2b protein, expressed from the subgenomic RNA 4A, is a suppressor of gene silencing, while RNA 3 encodes the 3a protein, necessary for viral movement, and the coat protein (CP) that is expressed from the subgenomic RNA 4 (Roossinck, 2002). CMV has the widest host range of any other plant virus and infects more than 1200 species, including monocots and dicots, herbaceous and woody plants (Edwardson and Christie, 1991; Zitter and Murphy, 2009). In addition, CMV can be transmitted by more than 80 aphid species (Palukaitis and García-Arenal, 2003). Because of this and its worldwide occurrence, CMV is economically very important. Based on phylogenetic analysis of the CP ORF and the 5' non-translated region (NTR) of RNA 3, as well as on biological, serological and molecular characteristics, CMV strains are subdivided into the main subgroups I and II (Owen and Palukaitis, 1988; Palukaitis and García-Arenal, 2003). Many CMV hosts are susceptible to both subgroups; therefore, the most favourable resistance should be efficient against both of them. In spite of the several natural resistance genes identified in different hosts (Jacquemond, 2012; Choi et al., 2018), there are no commercially available lines resistant to CMV.

Recently, recombinant antibodies (rABs) expressed in plants have been used successfully to confer resistance against plant viruses, without the perceived biosafety risks associated with

pathogen-derived resistance strategies (Thompson and Tepfer, 2010; Peschen et al., 2016). The first report of rAbs expressed *in planta* was against the CP of artichoke mottled crinkle virus (AMCV) (Tavladoraki et al., 1993). Using this approach, only partial resistance was achieved, possibly because of the relatively large amounts of CP accumulating in plants infected by this virus. More recently, broader and higher levels of resistance have been obtained using single-chain variable antibody fragments (scFvs). scFv molecules contain the variable light (V_L) and heavy (V_H) chains of an antibody, connected by a polypeptide that maintains the antigenic specificity of the complete molecule (Raag and Whitlow, 1995; Liu et al., 2015). scFv antibodies obtained by the "phage display" methodology and transgenically or transiently expressed in plants conferred resistance against different viruses, thanks to their specific interaction with various target viral antigens (Safarnejad et al., 2011). Plant resistance was reported to occur through the interaction with the RdRp of tomato bushy stunt virus (Boonrod et al., 2004), the nuclear inclusion (NIa) protein of potato virus Y (Gargouri-Bouzid et al., 2006; Ayadi et al., 2012), the P1 protein of potato leafroll virus (Nickel et al., 2008), the p25 major coat protein of citrus tristeza virus (Cervera et al., 2010), the NIb protein of plum pox virus (Gil et al., 2011), and the CP of banana bunchy top virus (Shilpa, 2013). This scFv-based resistance strategy resulted efficient also against other plant pathogens, such as phytoplasmas, fungi, bacteria, and viral vectors (Safarnejad et al., 2011 and 2013; Peschen et al., 2016). scFv fragments have also great biotechnological potential with wide medical applications and may be used for the preparation of immunotoxins, for therapeutic gene delivery and as biosensors (Ahmad et al., 2012; Crivianu-Gaita and Thompson, 2016).

Intracellular Antibody Capture Technology (IACT) provides the direct selection of scFv antibodies using the yeast two-hybrid system without having to rely on an *in vitro* system, such as the "phage display" technique (Visintin et al., 2004). While most efforts to engineer resistance to CMV have focused on transgenic approaches using viral sequences (pathogen-derived resistance, Prins et al., 2008; Morroni et al., 2008), there are few reports on the use of scFvs against the CP as transgenes, conferring resistance to CMV (Villani et al., 2005; Aebig et al., 2006). Furthermore, if

the CP is selected as target gene, the broadness of resistance could be hampered by its variability; this can be avoided by choosing a different viral protein, such as the polymerase, which is more conserved, expressed at lower levels, and fundamental at early stages of infection. Therefore, the objective of this work was to obtain transgenic tobacco plants (*Nicotiana tabacum*) transformed with a scFv selected using IACT against the 2a polymerase of CMV and to estimate the level of resistance against representative isolates of both CMV groups I and II. Interestingly, these scFvs were selected from a library initially generated against the unrelated non-structural rotavirus protein NSP5 (Vascotto et al., 2005).

2. Results

2.1. Yeast two-hybrid selection assays

All fusion constructs (pBTM116-2a, pBTM116-Motifs, and pBTM116-GDD) consisting of LexA and the RdRp-derived proteins of the CMV strain I17F (Group I) (Fig. 1) were checked for correct expression in the yeast reporter strain, using Western blot and an anti-LexA antibody. The LexA fusions were stably and properly expressed, except for the full-length 2a protein that could not be visualised possibly for its insufficient expression level or the inability to be recognized by the anti-LexA antibody (Fig. 2).

Of the three CMV RdRp-derived proteins tested, the GDD protein transactivated the two Histidine and LacZ reporter genes in the pBTM116 vector (not shown) and was therefore discarded from further analyses. In contrast, the full length 2a and the Motifs proteins interacted with 96 and 25 scFvs library prey constructs, respectively (Table 1), without unspecific activation of the LexA-CMV antigen. Among all the colonies obtained, 77% of the constructs against the full length 2a protein and 36% against the Motifs protein were confirmed positive for interaction in the ß-galactosidase filter assay. Four of these exhibited the strongest signal in the β-galactosidase assay (Fig. 3A) and displayed a different profile in fingerprinting analysis when digested with *Ava*II (Fig.

3B). Therefore scFvF6 and scFvF71 against the full length 2a protein and scFvM52 and scFvM181 against the Motifs protein were selected for further analysis (Table 1).

Amino acid alignment of these scFvs showed an identity in the range of 54-59% in the light-chain of the variable domain (V_L) and a higher variation in the heavy-chain of the variable domain (V_H), ranging from 34% (scFvM52 vs. scFvF71) to 80% (scFvM181 vs. scFvF6) (Fig. 3C, D, E). Nucleotide sequence analysis of the V region showed that the selected scFvs belong to different Ig germline families (Table 1), with the only exception of two V_H regions (scFvF6 and scFvM181) that belong to the same family (IGHV5), as a consequence of the highest amino acid similarity in this region.

2.2. Cloning of scFvs in pCAMBIA2300 for tobacco transformation

Stable transformation of tobacco plants was achieved by cloning the four scFvs into the binary vector pCAMBIA2300 and delivering the obtained constructs using *Agrobacterium tumefaciens* and leaf explants, under kanamycin selection (Table 2). To estimate whether selected scFvs conferred CMV resistance *in planta*, a total of 18 transgenic T_0 plants were regenerated, i.e. eight for the Motifs construct (2 for scFvM52 and 6 for scFvM181) and ten for the full length 2a construct (5 for scFvF6 and 5 for scFvF71). Out of these T_0 lines, 6 lines expressed a scFv transgene of approximately 30 kDa, i.e. lines 181.4 (expressing scFvM181), 718.1, 718.3, 718.4 (scFvF71), 62.4, 62.8 (scFvF6) (Fig. 4), with the strongest expression occurring in the transgenic line 718.1.

2.3. Interaction of CMV proteins and scFvs in mammalian cells

In order to analyse the cellular localization of the CMV antigens used as prey, the genes encoding the 2a and Motifs proteins were cloned in the pEGFP-N1 vector and expressed in mammalian cells as GFP fusions. Both the 2a and Motifs proteins were found mainly located in the cell cytoplasm, as determined by confocal immunofluorescence analysis (Fig. 5A), thought with a different pattern. CMV-Motifs-GFP formed aggregates probably due to the overexpression of the

exogenous protein (Kopito and Sitia, 2000), while CMV-2a-GFP was uniformly distributed (Fig. 5A). To determine their binding activity, each scFv construct was co-transfected with EGFP fusion constructs expressing the corresponding interacting CMV antigen, 2a or Motifs. Interestingly, when scFvM52 and scFvM181 were co-expressed with the Motifs protein (Table 2) they co-localized forming the same aggregates found with CMV-Motifs-EGFP alone, confirming the positive interaction between the two components (Fig. 5B). Conversely, the CMV full-length 2a protein did not form aggregates in the cytoplasm, making difficult to identify a clear interaction with its corresponding scFvF6 and scFvF71 in mammalian cells (data not shown).

2.4. CMV challenge of scFv transgenic plants

Following inoculation with the FNY strain of CMV (group I), we observed that all transgenic plants derived from line 718-4.2 were asymptomatic and accumulated a virus amount below the threshold limits when tested by ELISA at 2 wpi (Fig. 6 and 7). Plants of lines 62-4.3, 181-4.5, 718-1.2, 718-6.2 showed systemic symptoms and CMV accumulation above the threshold limits at 1 wpi, but when tested at 2 wpi the virus amount significantly decreased below the thresholds (Fig. 6). Furthermore, symptoms did not appear on newly emerged leaves by 3 wpi (Fig. 7). Overall, the percentage of infected plants in lines 62-8.1, 718-3.8, 718-5.2, 718-8.1, 528-4.5.6, and 528-6.2 was below or equal to 20% at 2 wpi (Suppl. Table 1), considerably lower than the results obtained at 1 wpi (Fig. 6).

When the same plants were challenged with the P132 isolate belonging to the CMV II group, transgenic lines 181-4.5, 181-7.4, 528-4.5.6, and 718-1.2 showed CMV level below the threshold limit (Fig. 7). Again, the level of CMV infection was noticeably reduced at 2 wpi compared to 1 wpi, and systemic symptoms strongly decreased at 3 wpi (Fig. 7).

4. Discussion

The intrabody-based *in vivo* protein knockdown strategy has been used successfully to develop plant virus resistance, as an alternative to other approaches such as RNA interference (Jaeger et al., 2000). The choice of the target virus protein, the subcellular localization of the antigen, and the level of expression of the intrabodies are the crucial points to consider to achieve efficient plant virus resistance. The first reports mainly used the CP as viral target of the antibodies and of scFvs (Villani et al., 2005). However, the concern that the CP diversity among plant viruses would negatively influence resistance coverage led to select more efficient antibodies and scFvs interacting with viral proteins with a higher level of amino acid conservation, such as the polymerase enzymes. Consequently, further reports relied on the high affinity of the antibodies to the polymerases of a few viruses, such as the antibody 5B-12B7 against the hepatitis C virus RdRp (Moradpour et al., 2002), the scFv against the tomato bushy stunt virus RdRp (Boonrod et al., 2004) and against the replication initiator protein of tomato yellow leaf curl virus (Safarnejad et al., 2009).

This work represents the first report on the use of scFvs selected against the CMV RdRp to obtain CMV resistance in transgenic tobacco plants. Notably, the scFvs identified in this work were initially detected for their interaction with the non-structural rotavirus protein NSP5; here, we demonstrated that these scFvs were not only successfully expressed in transgenic plants, but also interacted positively with the non-structural polymerase enzyme RdRp of the non-homologous virus CMV. There are very limited reports on scFvs binding to antigenic determinants of non-homologous proteins. A single variable domain of the shark immunoglobulin antibody was found to specifically interact with the hepatitis B virus pre-core antigen (Walsh et al., 2011). Our results demonstrate that specific interactions between the scFvs raised against an antigen of a non-structural protein of a human virus are possible also with an antigen deriving from a plant virus. This could be explained considering that the function of the NSP5 protein which is involved in the rotavirus replicative cycle (Vascotto et al., 2004). As the NSP5 protein used in the scFv screening strongly interacted with the rotavirus RdRp (VP1) (Arnoldi et al., 2007), this could also occur with

polymerases of other viruses, such as CMV, even if no relevant similarity was detected between rotavirus VP1 and CMV 2a protein sequences (not shown).

In this study, scFvF6 and scFvF71 that specifically interacted with the CMV full-length 2a protein and scFvM52 and scFvM181 that specifically bound the CMV Motifs (including the conserved motifs IV-VII of this protein) were selected. Since the GDD sequence positioned within the motif VI of 2a has been trans-activated, but a positive interaction was obtained with CMV Motifs, this interaction could be related to the presence of a specific epitope in the IV-V or VII subdomains of 2a, or to different epitopes distributed along the IV-VII domains. However, the importance of the full CMV 2a protein (and the possible involvement of other epitopes) should not be underestimated since the average protection level evaluated at 3 wpi against CMV was slightly higher for transgenic lines expressing scFvs against 2a (89%) than against Motifs (80%).

A previous report described the selection of a scFv from the phage display F8 library that specifically interacted with CMV virions in transgenic tomato plants, binding the virus in the inoculated leaves and preventing systemic infection and long distance movement (Villani et al., 2005). Compared to these plants, the transgenic tobacco plants of this study showed a slightly higher level of CMV resistance upon infection with the same CMV strain (FNY), as only 11% of the plants transformed with scFV against CMV 2a were susceptible, compared to 20% of the CMV CP-scFv plants. This result might be related to the choice of the antigen (RdRp vs. CP) or to the transformed host species (tobacco vs. tomato).

In spite of the difficulties to achieve scFvs accumulation in the cytosol due to the reducing environmental conditions that affect antibody folding and stability (Marschall et al., 2011), we could demonstrate that the intrabodies selected in this study were successfully expressed in the cytoplasm in a mammalian system, and this could presumably occur also in plants. The successful expression of scFv through correct antibody folding and disulfide bond-formation could be mediated by endoplasmic reticulum (ER) enzymes, such as the ER protein disulfide isomerase, as already reported (Ellgaard and Ruddock, 2005). Since the 2a protein is localized in the tonoplast of

infected tobacco cells (Cillo et al., 2002), a positive interaction of the two proteins might occur in the cytoplasm of tobacco cells.

Furthermore, a relationship between CMV resistance and scFv expression was noticed. In fact, all transgenic lines expressing the scFv at levels detectable by Western blot (Fig. 4) resulted the most resistant against both CMV I and II groups. This highlights the potential role of correct folding and expression of intrabodies in a proper cell compartment. Notably, this relationship was also important in conferring resistance to other plant viruses (Xu et al., 2006; Cervera et al., 2010). However, since resistance was obtained also in transgenic lines that did not show detectable levels of scFv expression (particularly lines 528-4.5 and 718-6.2), it might be that only small quantities of scFvs are necessary in the cytosol to induce resistance, as reported for plum pox virus resistance obtained with low accumulation levels of scFvs in plants (Gil et al., 2011).

In the inoculation assays with a CMV strain belonging to group I, which represents the more aggressive strains (Carrère et al., 1999), we assumed that the sufficient expression of scFv was reached in transgenic line 718.4.2 to successfully bind the CMV 2a protein and therefore interfere with CMV replication, conferring virus resistance and low virus titre. On the other hand, when plants were challenged with CMV group II, representing mild strains, the scFvs expressed in transgenic lines 718.1.2 and 181.4.5 were apparently sufficient to disarm CMV 2a and CMV-Motifs, respectively resulting in a low virus titre. Interestingly, the scFvs engineered into plants were initially selected against the 2a protein of CMV I subgroup, but were efficient also against a CMV strain of another subgroup. It would be interesting to evaluate the level of resistance of these plants when challenged with other cucumoviruses or with more distant members of the family *Bromoviridae*.

The phenotype of inoculated transgenic plants differed considerably between 1 and 3 wpi. In fact, all transgenic lines that were symptomatic at 1 wpi showed a decrease in CMV systemic symptoms within the following weeks, until complete symptom remission in five transgenic lines inoculated with the CMV strain of I group (181-4.5, 62-4.3, 718-1.2, 718-4.2, and 718-6.2). It is

possible that CMV resistance mediated by scFvs in transgenic tobacco affects the functionality of the 2a protein and thus impairs virus replication, but not encapsidation. One report highlights the possibility that CMV CP modulates the expression of the CMV 2b protein and antiviral silencing which causes symptom recovery in plants (Zhang et al., 2017). The presence of CP and the inhibition of the 2a protein function (preventing CMV replication and causing viral self-attenuation) could be related to the symptom recovery observed in transgenic tobacco plants at 2 wpi and to the subsequent symptom remission on newly emerged leaves. The possibility that the CP, whose expression is not attenuated by scFvs, modulates the antiviral silencing machinery might be one of the factors influencing the recovery of the infected plants, which requires further investigations.

4. Experimental

4.1. Constructs

A LexA-based yeast two-hybrid (Y2H) system containing pBTM116 as bait plasmid and pVP16 as prey plasmid was employed (Visintin et al., 1999). Bait constructs pBTM116-2a, pBTM116-Motifs, and pBTM116-GDD were engineered by cloning different fragments of the 2a protein coding gene of the CMV strain I17F (subgroup I), consisting of either the full-length 2a gene (839 aa), a fragment named 'Motifs' (132 aa), covering conserved motifs (IV-VII), and a fragment named 'GDD' (22 aa), covering the GDD conserved motif (VI) complex (Fig. 1A). All sequences were fused to the LexA fragment of the pBTM116 vector (Fig. 1B). PCR was performed using primers specific for the CMV selected sequences: (i) 334for (5' CGGGATCCGTATGGCTTTCCCTGCCCCCGCATTC 3') and 335rev (5' CGCTGCAGTCAGACTCGGGTAACTCCGCCACGTTC 3') for '2a'; (ii) 252for (5' CGGAATTCGATCTGTCTAAGTTTGATAAGTCTC 3') and 336rev: (5' CGCTGCAGTTACTTCGAACAAATATATGGTACGGCA 3') for 'Motifs'; (iii) GDD+ (5' CGGAATTCACCGACCAATTCGAAAAGCT 3') and GDD- (5' CGCTGCAGTTAAGGGGGAAGCAGTGAAAATC 3') for the 'GDD' fragment. Primers included

endonuclease restriction sites (underlined) (*Bam*HI, *Pst*I, *Eco*RI, *Pst*I, *Eco*RI, and *Pst*I, respectively) for cloning purposes. PCR conditions were 94°C for 5 min for denaturation, followed by 35 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 5 min, and a final extension step of 10 min at 72°C. Bait constructs pBTM116-2a, pBTM116-Motifs, and pBTM116-GDD were obtained by cloning amplicons obtained with the above primers, which resulted in fusion to the LexA fragment flanked by an ADH1 transcription promoter and terminator of pBTM116 vector. Besides, pBTM116-NSP5 containing the non-structural protein NSP5 of rotavirus (Vascotto et al., 2005) was used as a positive control.

A mouse scFv library produced for the NSP5 protein *Human rotavirus A* in the prey vector pVP16/D (Fig. 1C and 1D), containing 4 x 10^4 colonies with 95% diversity (Vascotto et al., 2005) was used in the Y2H system to identify positive interactions with the above CMV proteins. Vascotto et al. (2005) reported various intrabodies that were unable to interact with the rotavirus NSP5 protein in the cytoplasm of transfected mammalian cells. Because of their high diversity, it was assumed that that they could be used to screen for specific interactions against other proteins.

4.2. Yeast two-hybrid selection assays

All Y2H assays were done as described in previously published protocols, using the *Saccharomyces cerevisiae* reporter strain L40 (MATa his3–200 trp1–901 leu2–3, 112 ade2 LYS2::$(lexAop)_4$-HIS3 URA::$(lexAop)_8$-lacZ GAL4 gal80) (Vojtek et al., 1993). All LexA-fusion constructs (pBTM116-2a, pBTM116-Motifs, and pBTM116-GDD) were checked by Western blot for correct expression in the yeast reporter strain. For this, an overnight yeast culture was diluted in YC medium (Clontech, Mountain View, CA, USA) at OD600 =0.15 and grown at 30°C up to OD600 = 0.5–0.7. One ml of culture was centrifuged at 10,000 x *g* for 5 min and the cell pellet denatured by boiling in Laemmli buffer, resolved on 12% SDS-PAGE, and transferred onto a PVDF membrane (Millipore, Burlington, MA, USA). For antigen visualization, anti-LexA

polyclonal antibody at 1:2.000 dilution (ThermoFisher Scientific, Waltham, MA, USA), followed by anti-rabbit-AP antibody (ThermoFisher Scientific) were used.

S. cerevisiae L40 containing the bait plasmid (pBTM116-2a, pBTM116-Motifs, pBTM116-GDD, pBTM116-NSP5) was transformed with pVP16/D-scFv and grown on selective medium, followed by a β-galactosidase filter assay, as described by Visintin et al (1999). Four selected scFvs which interacted with the CMV RdRp (scFvM52 and scFvM181 with CMV Motifs, and scFvF6 and scFvF71 with CMV full-length 2a) were cloned into different expression vectors for further investigations (pCAMBIA2300 and pEGFP-N1).

4.3. Cloning of scFvs in pCAMBIA2300 for stable tobacco transformation

For cloning in the binary vector pCAMBIA2300 (Cambia, Canberra, Australia), PCR was performed with primers specific for the four selected scFvs, containing the SV5-tag at the 3' terminus of the heavy-chain variable domains (V_H), i.e. 52_71F (5' CATCGATACAATGGCCCATGCCGACATTCAGATG 3') and 52R (5' CATCGATTTAGGTAGAATCGAGGCCGAGGAGAGGGTTAGGGATAGGCTTGCCTGCAGA GACAGTGACCAGAGTCCCTTGGCC 3') for scFvM52, 52_71F (5' CATCGATACAATGGCCCATGCCGACATTCAGATG 3') and 181R (5' CATCGATTTAGGTAGAATCGAGGCCGAGGAGAGGGTTAGGGATAGGCTTGCCTGAGGA GACGGTGACTGAGGTCCCTGCGCC 3') for scFvM181, 6F (5' CATCGATACAATGGCCCATGCCGATATTGTAATG 3') and 6R (5' CATCGATTTAGGTAGAATCGAGGCCGAGGAGAGGGTTAGGGATAGGCTTGCCTGAGGA GACGGTGACCGTGGTGCCTTGGCC 3') for scFvF6, and 52_71F (5' CATCGATACAATGGCCCATGCCGACATTCAGATG 3') and 71R(5'CATCGATTTAGGTAGAATCGAGGCCGAGGAGAGGGTTAGGGATAGGCTTGCCTG AGGAGACTGTGAGAGTGGTGCCCTGGCC 3') for scFvF71.

PCR products were cloned in-frame into the *Nru*I restriction site of pCAMBIA2300, modified with the expression cassette of the pFF19 vector under the control of the enhanced cauliflower mosaic virus 35S promoter. Cloned vectors were introduced into *A. tumefaciens* strain C58 by freeze/thaw transformation, according to An et al., 1988.

4.4. Transformation of N. tabacum

Stably transformed tobacco plants (*N. tabacum* var. *Xanthi*) were generated by leaf disc transformation with recombinant *A. tumefaciens,* essentially as described by Horsch et al., 1985. Transgenic plants were grown in greenhouse conditions with a 16/8 h (light/dark) photoperiod. Expression of scFv was determined by Western blot on total proteins extracted from transgenic tissue after homogenization in Laemmli sample buffer (approximately 9 µl/mg tissue). The western blot was developed using an anti-SV5 MAb diluted at 1:5000, followed by anti-mouse HRP Ab (KPL, SeraCare, Milford, MA, USA).

4.5. Cloning of CMV genes into pEGFP-N1 and scFvs into pcDNA3 and their expression in mammalian cells

cDNA sequences encoding the CMV 2a and Motifs proteins were amplified using the following primers: 409for (5' CGCTGCAGGCCACCATGGCTTTCCCTGCCCCCGCATTC 3') and 410rev (5'CG**GGATCC**GACTCGGGTAACTCCGCCACGTTC 3') for 2a, and 411for (5'CG**GAATTC**TGGCCACCATGGATCTGTCTAAGTTTGATAAGTCTC 3') and 253rev (5' CGCTGCAGCTTCGAACAAATATATGGTACGGCA 3') for Motifs. PCR CMV 2a and Motifs products were cloned into *Pst*I (underlined) and *Bam*HI (bold) of pEGFP-N1 (Clontech) vector, fused in frame with the enhanced green fluorescent protein (EGFP) gene.

scFvs in pVP16/D were digested with *Nhe*I and *Hind*III restriction enzymes and cloned into the same restriction fragment of the pcDNA3 vector (ThermoFisher Scientific). pcDNA3-M52,

pcDNA3-M181, pcDNA3-F6, and pcDNA3-F71 constructs were tagged with two nuclear localization signals (NLS) of SV40 T-antigen within the *Nhe*I restriction site using annealed oligonucleotides, as described by Vascotto *et al*. (2005).

4.6. Mammalian cells culture and transfection

Rhesus monkey kidney MA104 cells were routinely cultured in Dulbecco's modified Eagle's medium containing 10% foetal calf serum (Gibco, ThermoFisher Scientific). Cell cultures maintained in the absence of serum and antibiotics (serum-free medium) were used for DNA transfections. Transient transfections with vaccinia virus were performed as previously described (Eichwald et al., 2002). One tenth of the volume of total cellular extracts was used in Western blot analysis. For cells transfected with scFvs, an anti-SV5 MAb diluted at 1:5000, followed by anti-mouse HRP Ab (KPL) were used, while for cells transfected with CMV 2a and Motifs, an anti-GFP rabbit polyclonal Ab (ThermoFisher Scientific) diluted at 1:2000, followed by anti-rabbit HRP Ab (ThermoFisher Scientific) were used.

4.7. Immunofluorescence microscopy

Transfected cells were washed twice with phosphate buffered saline (PBS) and fixed with 3.7% paraformaldehyde for 10 min at room temperature. After fixation, the cells were washed three times with PBS and permeabilised with 0.1% Triton in PBS for 5 min. Next, samples were washed with PBS, and non-specific binding sites were blocked with 1% BSA in PBS for 30 min. Slides were incubated with anti-SV5 MAb and anti-GFP Ab for 1 h in a moist chamber. Anti-mouse RITC-conjugated antibody (Pierce, Rockford, IL, USA) was used at a 1:600 dilution. Thereafter, samples were washed and mounted using ProLong mounting medium (Molecular Probes, Eugene, OR, USA). Images were acquired with an argon–helium double laser confocal microscope (Zeiss, Oberkochen, Germany).

4.8. Virus challenge of scFv transgenic tobacco plants

T2 tobacco plants were grown in soil and maintained in greenhouse conditions at 20–28/16–20◦C (day/night), with and a 16/8 h (light/dark) photoperiod. Individual plantlets with three fully developed leaves were dusted with abrasive powder (Carborundum, Sigma, Kawasaki, Japan) and inoculated by rubbing the upper surface with fingers dipped in the virus inoculum. This consisted of extracts from young symptomatic leaves of *N. benthamiana* infected by the CMV FNY or P132 isolates, or of a healthy plant, ground in ice-cold inoculation buffer (10 mM phosphate buffer, pH 7.0, 5 mM Na-diethydithiocarbamate, 1 mM EDTA, and 5 mM thioglycolic acid-Na salt). Non-transformed tobacco plants var. Xanthi were used as positive control plants. Inoculated plants (n=6) were maintained for up to 6 weeks post-inoculation for symptom scoring.

4.9. Double-Antibody Sandwich/Enzyme linked immunosorbent assay (DAS-ELISA)

DAS-ELISA (Clark and Adams, 1977) was performed for virus detection using leaf extracts homogenized in PBS-T at a 1 mg/ml ratio. The polyclonal mix antibody (PAb) DTL ToRS (Loewe Biochemica GmbH, Germany) reacting against both CMV groups I and II was used at 1:1000 dilution. Results are expressed as mean OD450 nm values of each of the six inoculated plants, tested in triplicate. Non-inoculated transgenic plant were used as negative controls and plants with values at least three times greater than negative controls were considered positive.

5.0. Statistical analysis

Data from DAS-ELISA assay were submitted to analysis of variance (ANOVA) by using the Statistical Package for Social Science (SPSS, version 17.0, IBM, Chicago, IL, USA). Statistical significance ($p < 0.05$) was determined using the *t* test for transgenic lines against the non-transformed tobacco plants used as positive controls.

5.1. Conclusion

In conclusion, from an NSP5 scFv library, we selected four intrabodies that positively interacted with the full-length 2a and the 2a conserved motif region of the CMV polymerase. Following their transformation in tobacco, we observed that they conferred resistance to both CMV subgroups I and II. Higher resistance was achieved with intrabodies targeting the full-length 2a protein, highlighting the possibility that more than one epitope is involved in the positive protein interactions required to confer CMV resistance.

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Competing interests

The authors declare they have no competing interests.

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Figure Legends

Figure 1. Yeast two-hybrid screening of CMV proteins interacting with the rotavirus NSP5-scFv library. (A) Schematic representation of the CMV genome. The proteins considered in this work, encoded by RNA 2, are the full length 2a protein (RdRp, yellow), the Motifs domain (light orange), and the GDD motif domain (dark orange). (B) Scheme of pVP16/D plasmid used for constructing the scFv library. NLS, nuclear localisation signal, V_L and V_H, light- and heavy-chain variable domains, VP16, trans-activating protein; Maps of (C) the pBTM116 plasmid carrying CMV proteins and (D) the pVP16/D plasmid containing the scFv library.

Figure 2. Expression of the LexA-CMV protein fusions in yeasts, analysed in Western blot with anti-LexA antibody. (A) M, Prestained protein marker (in kDa); 1, Empty pBTM116 (LexA, 24 kDa); 2, pBTM116-2a (121 kDa); 3, pBTM116-Motifs (39 kDa). (B) M, Kaleidoscope protein marker (in kDa); 1, Empty pBTM116 (LexA, 24 kDa); 2, pBTM116-GDD (26 kDa).

Figure 3. Characterization of scFvs positively interacting with CMV proteins. (A) β-galactosidase assay on yeast colonies co-expressing the scFvs M52 and M181 interacting with CMV Motifs and the scFvs F6 and F71 interacting with the full length CMV 2a, grown on selective plates lacking uracil, tryptophan, histidine, leucine, and lysine. The pBTM116-NSP5 construct with the pVP16/D-scFv construct represents the positive control, while the pBTM116-NSP5 construct with pVP16/D construct is the negative control. (B) Fingerprinting analysis (*Ava*II digestion) of pVP16 containing the selected scFVs. The arrows indicate the DNA fragment size. (C) Amino acid sequence alignment of the V_L and V_H domains, including the linker region (amino acid positions 101-132). (D, E) Amino acid identity of scFvs in the V_L and the V_H regions, respectively.

Figure 4. Western blot analysis of scFv expression in transgenic *N. tabacum* lines, showing representative lines transformed with the different scFv constructs, i.e. line 528.4 (scFvM52), 181.4

(scFvM181), 62.4 (scFvF6), and 718.6, 718.4, 718.1 (scFvF71). The asterisks indicate the expressed scFv. M, Kaleidoscope protein standards (in kDa); wt, wild-type plants.

Figure 5. Confocal immunofluorescence analysis of mammalian cells (A) transfected with the selected CMV-EGFP proteins or (B) co-transfected with the control scFv-NLS (red) or with the target CMV Motifs-EGFP protein (green).

Figure 6. Mean CMV accumulation in T2 transgenic plants (n=6) inoculated with the CMV FNY (group I) (A, B) or the P132 isolate (group II) (C, D), evaluated by DAS-ELISA at 1 and 2 weeks post-inoculation (wpi). White and grey represent lines transformed with the '2a' or 'Motif' constructs, respectively. Non-transformed tobacco plants var. Xanthi were used as positive controls (black). Bars represent standard errors. The significance ($p < 0.05$) between transgenic lines and wt control line is indicated by an asterisk. The dashed line indicates the arbitrarily defined threshold to consider a plant as infected.

Table 1.

Selection and characterization of scFv library in the yeast two-hybrid assays for positive interactions with CMV '2a' and 'Motifs' proteins.

CMV protein target	N. of interacting intrabodies	Selection in yeasts		N. of lines with different pattern after *Ava*II restriction	Name of selected scFvs	Ig germline family	
		β-gal expression	Growth on Y(-L) but not Y(-WL)			V_L	V_H
2a	96	74	100%	2	F6	KV4-59	HV5-6
					F71	KV14-111	HV8-12
Motif	25	9	80%	2	M52	KV6-25	HV1-47
					M181	KV3-12	HV5-17

Table 2.

Selection of transgenic lines and interaction of selected scFvs with their target CMV protein.

CMV protein target	scFv clone	Formation of cytoplasmic aggresomes in mammalian cells	N. of T_0 lines obtained	N. of lines expressing a 30-kDa protein (name of line)
2a	F6	ND	5	2 (62.4, 62.8)
	F71	ND	5	3 (718.1, 718.3, 718.4)
Motif	M52	Yes	2	0
	M181	Yes	6	1 (181.4)

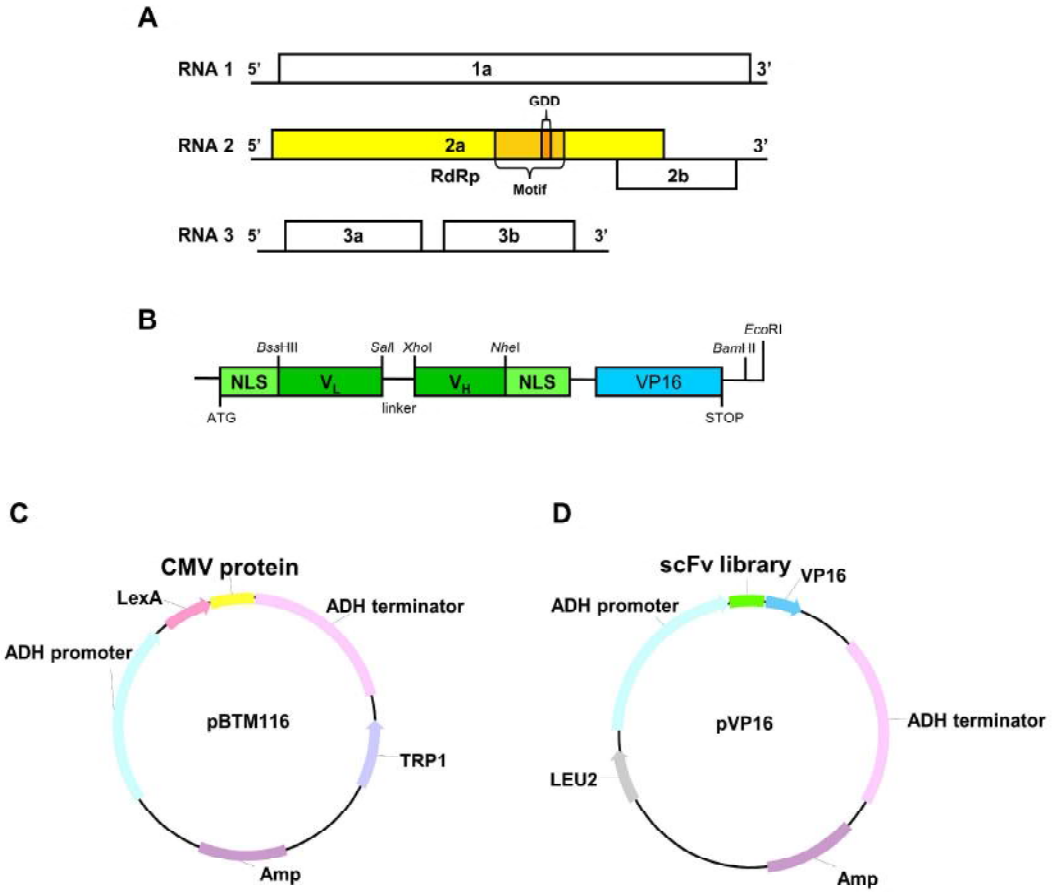

Figure 1
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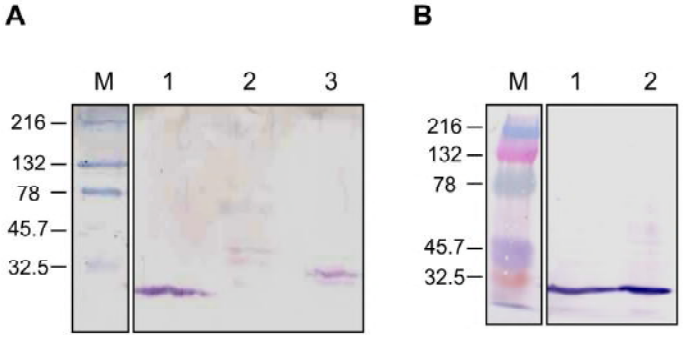

Figure 2
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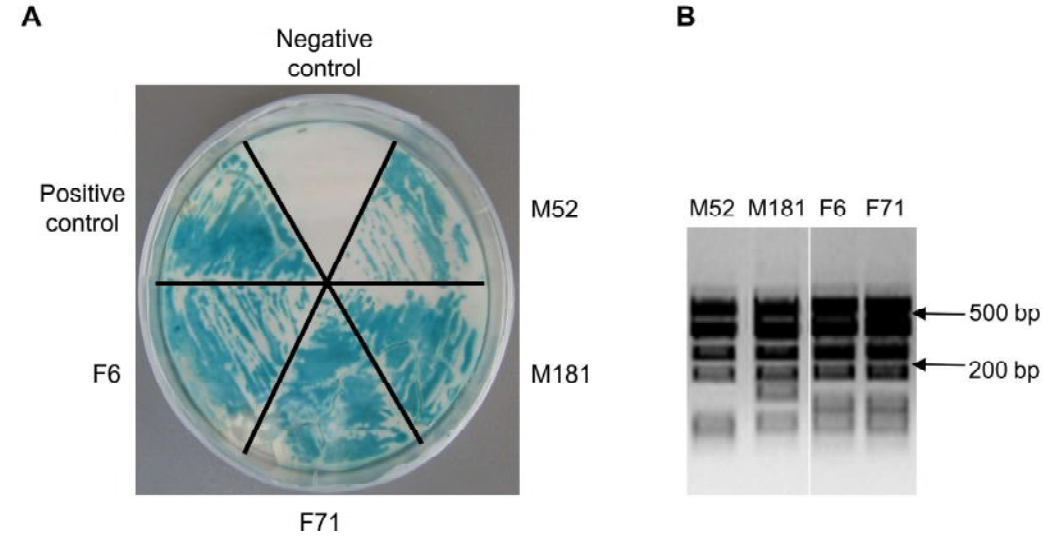

C

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                1                                                   100
scFvF6    (1)   DIVMTQSPAIMSASPGEKVTMTCSASSSVS-----YMHWYQQKSGTSPKRWIYDTSKLASGVPARFSGSGSCTSYSLTISSMEAEDAATYYCQQWSSNPL
scFvM181  (1)   DIQMMQSPASLAVSLGQRATISCRASKSVSTSGYSYMHWYQQKPGQPPKLLIYLASNLESGVPARFSGSGSGTDFTLNIHPVEEEDAATYYCQHSRELPF
scFvF71   (1)   DIQMLQSPSSMYASLGERVTITCKASQDIN----SYLSWFQQKPGKSPKTLIYRANRLVDGVPSRFSGSGSGQDYSLTISSLEYEDMGIYYCLQYDEFPF
scFvM52   (1)   DIQMNQSHKFMSTSVGDRVSITCKASQDVS----TAVAWYQQNPGQSPKLLIYWASTRHTGVPDRFTGSGSGTDYTLTISSVQADDLALYYCQQHYSTPY
Consensus (1)   DIQMIQSPASMSASLGERVTITCKASQSVS    SYMHWYQQKPGQSPKLLIY ASKL SGVPARFSGSGSGTDYSLTISSVEAEDAATYYCQQW S PF
                101                                                 200
scFvF6    (96)  TFGAGTKLELKRSGGSTSGSGKPGSGEGSSGTEVMLVESGGGLVQPGGSRKLSCAASGFTFSS--YGMSWVRQTFDKRLEWVATISSGGSYTYYPDSVKG
scFvM181  (101) TFGSGTKLEIKRSGGSTSGSGKPGSGEGSSGTEVMLVESGGGLVQPGGSRKLSCAASGFTFSS--FGMHWVRQAPEKGLEWVAYISSGSSTIYYADTVKG
scFvF71   (97)  TFGAGTKLELKRSGGSTSGSGKPGSGEGSSGTQVTLKVSGPGILQPSQTLSLTCSFSGFSLSTSGMGVSWIRQPSGKGLEWLAHIY-WDDDKRYNPSLKS
scFvM52   (97)  TFGGGTKLEIKRSGGSTSGSGKPGSGEGSSGTEVKLMESGAELVKPGASVKMSCKAFGYTFT--YYIEWMKQNHGKSLEWIGNFHPYNDDTKYNEKFKG
Consensus (101) TFGAGTKLEIKRSGGSTSGSGKPGSGEGSSGTEVMLVESGGGLVQPGGSRKLSCAASGFTFSS  YGMSWVRQ PGKGLEWVAHISSG SDTYYNDSVKG
                201                                 254
scFvF6    (194) RFTISRDNAKNNLYLQMSSLKSEDTAMYYCTRESDYGNSFYFDVWGQGTTVTVSS
scFvM181  (199) RFTISRDNPKNTLFLQMSSLKSEDTAMYYCARRGS-SDWYFDVWGAGTSVTVSS
scFvF71   (196) RLTISKDTSRMQVFLKIANVDTADSATYYCARIFD--YDHFDYWGQGTTLTVSS
scFvM52   (195) KAKLTVEKSSSTVYLELSRLTSDDSAVYYCARGG-----FA-YWGQGTLVTVSA
Consensus (201) RFTISRDNSKNTLFLQMSSLKSEDSAMYYCAR GD   FYFDYWGQGTTVTVSS
```

D

scFv	M181	F6	M52	F71
M181		58	54	54
F6			59	59
M52				59
F71				

V_L

E

scFv	M181	F6	M52	F71
M181		80	42	43
F6			43	44
M52				34
F71				

V_H

Figure 3
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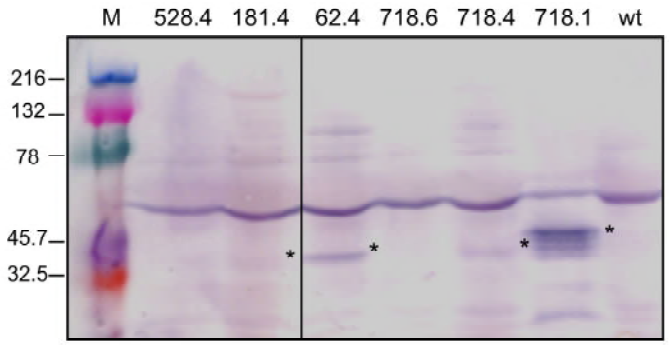

Figure 4
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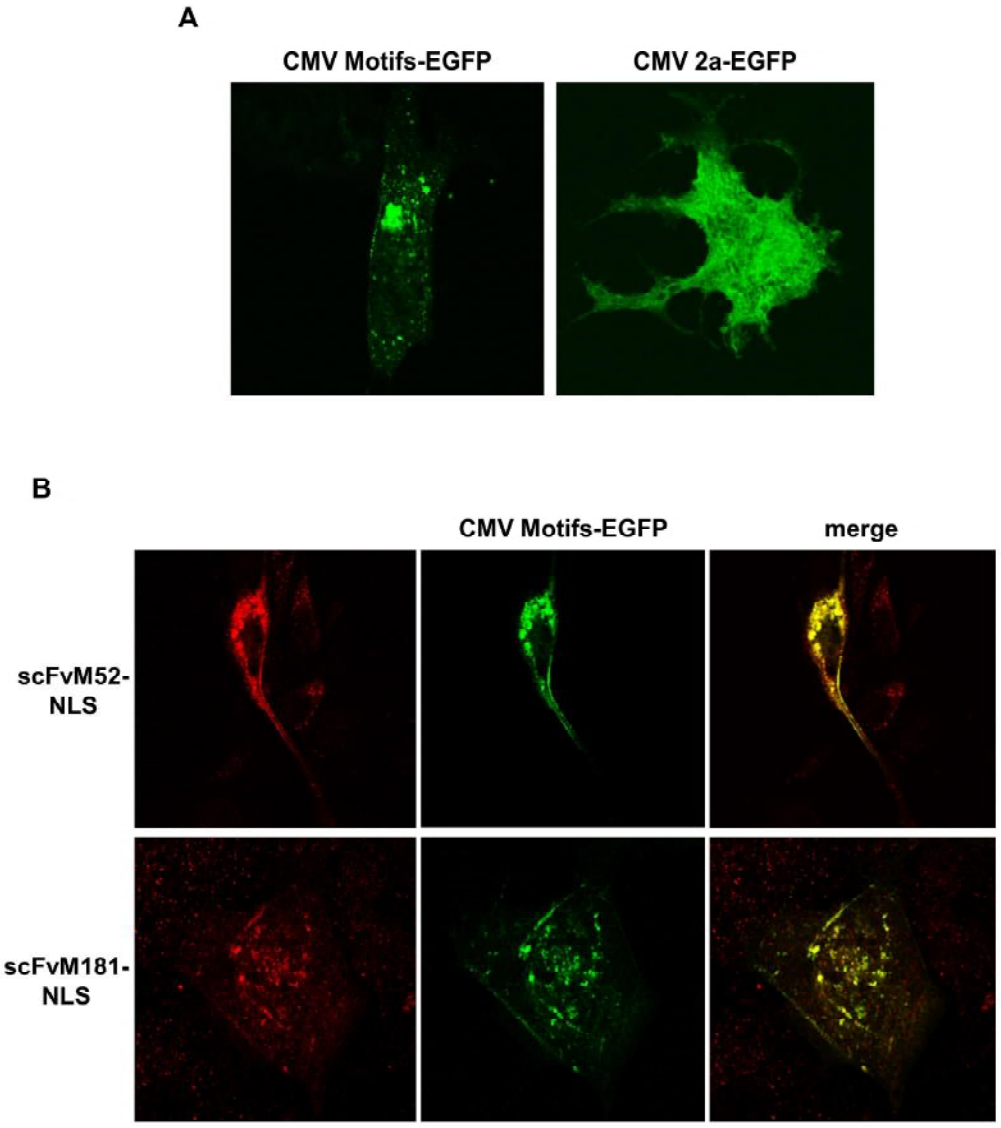

Figure 5
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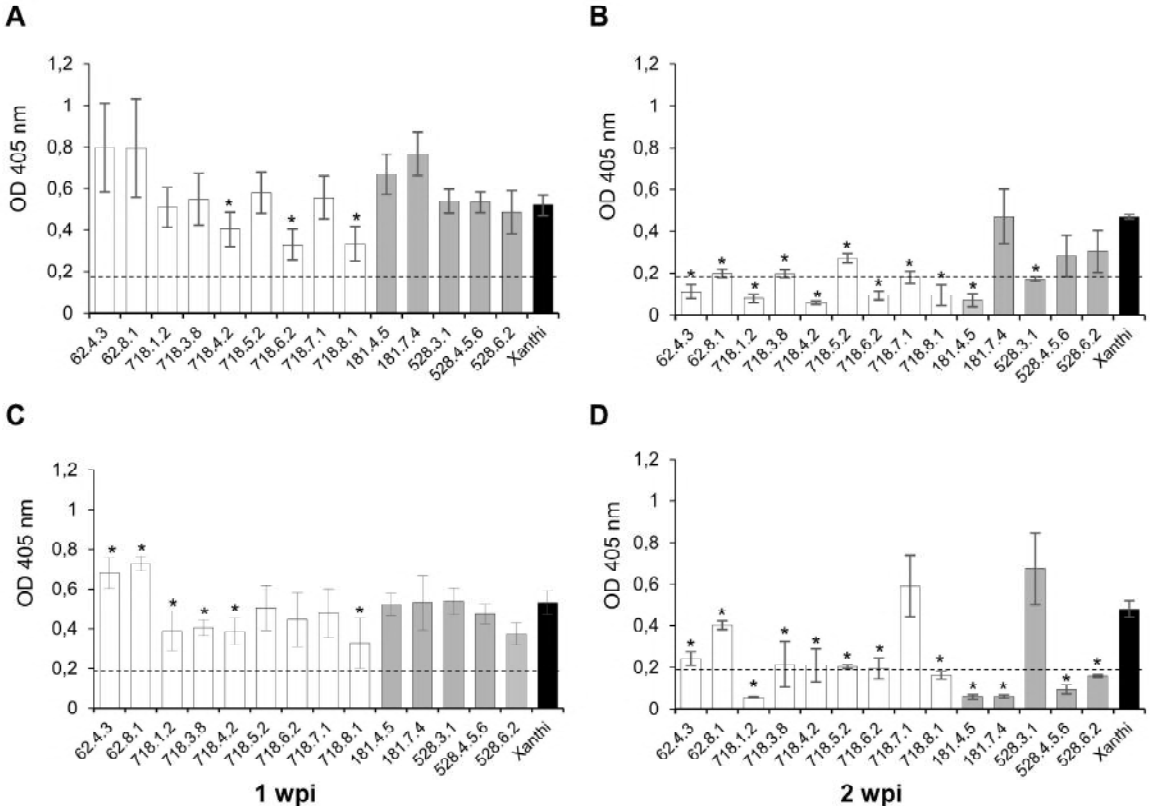

Figure 6
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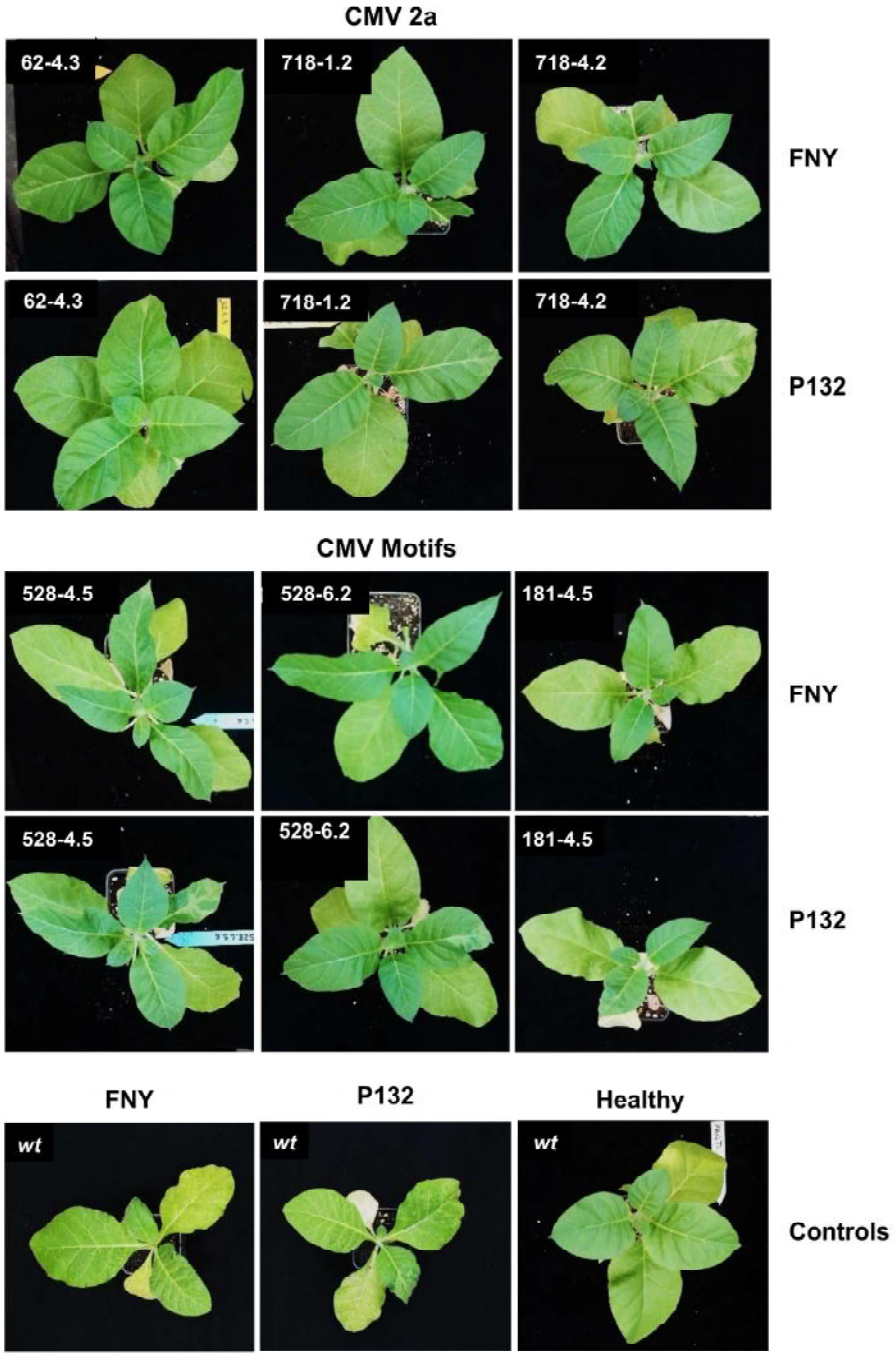

Figure 7
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