

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Engineering partial resistance to cucumber mosaic virus in tobacco using intrabodies specific for the viral polymerase

This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1730430> since 2020-02-24T14:38:51Z

Published version:

DOI:10.1016/j.phytochem.2019.03.006

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

1
2
3
4 1
5
6
7 2 **Engineering partial resistance to cucumber mosaic virus in tobacco using**
8
9 3 **intrabodies specific for the viral polymerase**
10
11
12 4

13
14
15 5 **Slavica Matic^{ab,*}, Emanuela Noris^{b,*}, Roberta Contin^c, Daniele Marian^b, Jeremy R.**
16
17
18 6 **Thompson^{ad,**}**
19
20
21 7

22
23
24 8 *^a Plant Virology Group, ICGEB Biosafety Outstation, Ca' Tron di Roncade (TV), Italy*

25
26 9 *^b Institute for Sustainable Plant Protection, National Research Council of Italy (IPSP-CNR), Turin (TO),*
27
28 10 *Italy*

29
30 11 *^c International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste (TS), Italy*

31
32 12 *^d Department of Plant Pathology and Plant-Microbe Biology, Cornell University, Ithaca, USA*
33
34 13
35
36 14
37
38 15
39
40 16

41 17 * These authors contributed equally to this work
42
43 18

44
45 19 ** Corresponding author. Department of Plant Pathology and Plant-Microbe Biology, 334 Plant Science
46
47 20 Building, Cornell University, Ithaca, NY 14853, USA. Tel +1-607-255-0872; Fax +1-607-255-4471
48

49 21 *E-mail address: jrt36@cornell.edu (J. R. Thompson)*
50
51 22
52
53 23
54
55 24
56
57
58
59

60
61
62 25 **ABSTRACT**
63

64 26 A single-chain variable antibody fragment (scFv) library tested against the non-structural NSP5
65 27 protein of human rotavirus A was screened by a yeast two-hybrid system against three proteins
66 28 derived from the RNA-dependent RNA polymerase (RdRp) of cucumber mosaic virus (CMV), with
67 29 the aim of blocking their function and preventing viral infection once expressed *in planta*. The
70 30 constructs tested were (i) '2a' consisting of the full-length 2a gene (839 amino acids, aa), (ii)
71 31 'Motifs' covering the conserved RdRp motifs (IV-VII) (132 aa) and (iii) 'GDD' located within the
72 32 conserved RdRp motif VI (GDD, 22 aa). The '2a' and 'Motifs' constructs interacted with 96 and 25
73 33 library constructs, respectively, while the 'GDD' construct caused transactivation. The scFvs
74 34 positive in yeast two-hybrid system were analyzed *in vivo* for their interaction with the 2a and
75 35 Motifs proteins in a mammalian transient expression system. Eighteen tobacco lines stably
76 36 transformed with four selected scFvs were produced and screened for resistance against two
77 37 different CMV isolates. Different levels of resistance and rate of recovery were observed with CMV
78 38 of both groups I and II, particularly in lines expressing intrabodies against the full-length 2a protein.
79 39 This work describes for the first time the use of intrabodies against the RdRp of CMV to obtain
80 40 plants that reduce infection of a pandemic virus, showing that the selected scFvs can modulate virus
81 41 infection and induce premature recovery in tobacco plants.
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99

100 43 **Keywords:** *Nicotiana tabacum*; *Solanaceae*; tobacco; *Cucumber mosaic virus* (CMV); yeast two-
101 44 hybrid system; transgenic plants; virus resistance; intrabodies; scFv; RNA-dependent RNA
102 45 polymerase (RdRp).
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118

119
120
121 **1. Introduction**
122

123 48 *Cucumber mosaic virus* (CMV) is the type species of the genus *Cucumovirus*, family
124 49 *Bromoviridae* (Roossinck, 1999). It is an icosahedral virus with an approximate diameter of 30 nm.
125 49 The CMV genome consists of three single-stranded genomic RNAs (RNAs 1, 2, and 3). RNAs 1
126 49 and 2 encode the 1a and 2a proteins, respectively (Jacquemond, 2012), which together form the
127 50 viral replicase, part of the putative replication complex shown to localize to the tonoplast (Hayes
128 50 and Buck, 1990; O'Reilly et al., 1998; Cillo et al., 2002). The 2a protein is the RNA-dependent
129 50 RNA polymerase (RdRp). RdRps have highly conserved amino acid motifs (Koonin, 1991; O'Reilly
130 51 and Kao, 1998) and a structure conserved even among distantly related viruses, consisting of “palm,
131 51 thumb and finger” structural domains with four common amino acid motifs (A-D), including the
132 52 GDD sequence in motif C, for magnesium co-ordination. The 2b protein, expressed from the
133 52 subgenomic RNA 4A, is a suppressor of gene silencing, while RNA 3 encodes the 3a protein,
134 53 necessary for viral movement, and the coat protein (CP) that is expressed from the subgenomic
135 53 RNA 4 (Roossinck, 2002). CMV has the widest host range of any other plant virus and infects more
136 54 than 1200 species, including monocots and dicots, herbaceous and woody plants (Edwardson and
137 54 Christie, 1991; Zitter and Murphy, 2009). In addition, CMV can be transmitted by more than 80
138 54 aphid species (Palukaitis and García-Arenal, 2003). Because of this and its worldwide occurrence,
139 54 CMV is economically very important. Based on phylogenetic analysis of the CP ORF and the 5'
140 54 non-translated region (NTR) of RNA 3, as well as on biological, serological and molecular
141 54 characteristics, CMV strains are subdivided into the main subgroups I and II (Owen and Palukaitis,
142 54 1988; Palukaitis and García-Arenal, 2003). Many CMV hosts are susceptible to both subgroups;
143 54 therefore, the most favourable resistance should be efficient against both of them. In spite of the
144 54 several natural resistance genes identified in different hosts (Jacquemond, 2012; Choi et al., 2018),
145 54 there are no commercially available lines resistant to CMV.
146 54
147 54
148 54
149 54
150 54
151 54
152 54
153 54
154 54
155 54
156 54
157 54
158 54
159 54
160 54
161 54
162 54
163 54
164 54
165 54
166 54
167 54
168 54
169 54
170 54
171 54

172 54 Recently, recombinant antibodies (rABs) expressed in plants have been used successfully to
173 54 confer resistance against plant viruses, without the perceived biosafety risks associated with
174 54
175 54
176 54
177 54

178
179
180 73 pathogen-derived resistance strategies (Thompson and Tepfer, 2010; Peschen et al., 2016). The first
181
182 74 report of rAbs expressed *in planta* was against the CP of artichoke mottled crinkle virus (AMCV)
183
184 75 (Tavladoraki et al., 1993). Using this approach, only partial resistance was achieved, possibly
185
186 76 because of the relatively large amounts of CP accumulating in plants infected by this virus. More
187
188
189 77 recently, broader and higher levels of resistance have been obtained using single-chain variable
190
191 78 antibody fragments (scFvs). scFv molecules contain the variable light (V_L) and heavy (V_H) chains
192
193 79 of an antibody, connected by a polypeptide that maintains the antigenic specificity of the complete
194
195 80 molecule (Raag and Whitlow, 1995; Liu et al., 2015). scFv antibodies obtained by the “phage
196
197 81 display” methodology and transgenically or transiently expressed in plants conferred resistance
198
199 82 against different viruses, thanks to their specific interaction with various target viral antigens
200
201
202 83 (Safarnejad et al., 2011). Plant resistance was reported to occur through the interaction with the
203
204 84 RdRp of tomato bushy stunt virus (Boonrod et al., 2004), the nuclear inclusion (NIa) protein of
205
206 85 potato virus Y (Gargouri-Bouزيد et al., 2006; Ayadi et al., 2012), the P1 protein of potato leafroll
207
208 86 virus (Nickel et al., 2008), the p25 major coat protein of citrus tristeza virus (Cervera et al., 2010),
209
210 87 the NIb protein of plum pox virus (Gil et al., 2011), and the CP of banana bunchy top virus (Shilpa,
211
212 88 2013). This scFv-based resistance strategy resulted efficient also against other plant pathogens, such
213
214 89 as phytoplasmas, fungi, bacteria, and viral vectors (Safarnejad et al., 2011 and 2013; Peschen et al.,
215
216 90 2016). scFv fragments have also great biotechnological potential with wide medical applications
217
218 91 and may be used for the preparation of immunotoxins, for therapeutic gene delivery and as
219
220 92 biosensors (Ahmad et al., 2012; Crivianu-Gaita and Thompson, 2016).

221
222
223 93 Intracellular Antibody Capture Technology (IACT) provides the direct selection of scFv
224
225 94 antibodies using the yeast two-hybrid system without having to rely on an *in vitro* system, such as
226
227 95 the “phage display” technique (Visintin et al., 2004). While most efforts to engineer resistance to
228
229 96 CMV have focused on transgenic approaches using viral sequences (pathogen-derived resistance,
230
231 97 Prins et al., 2008; Morroni et al., 2008), there are few reports on the use of scFvs against the CP as
232
233 98 transgenes, conferring resistance to CMV (Villani et al., 2005; Aebig et al., 2006). Furthermore, if

237
238
239 99 the CP is selected as target gene, the broadness of resistance could be hampered by its variability;
240
241 100 this can be avoided by choosing a different viral protein, such as the polymerase, which is more
242
243
244 101 conserved, expressed at lower levels, and fundamental at early stages of infection. Therefore, the
245
246 102 objective of this work was to obtain transgenic tobacco plants (*Nicotiana tabacum*) transformed
247
248 103 with a scFv selected using IACT against the 2a polymerase of CMV and to estimate the level of
249
250 104 resistance against representative isolates of both CMV groups I and II. Interestingly, these scFvs
251
252 105 were selected from a library initially generated against the unrelated non-structural rotavirus protein
253
254 106 NSP5 (Vascotto et al., 2005).

258 108 **2. Results**

260 109 *2.1. Yeast two-hybrid selection assays*

262
263 110 All fusion constructs (pBTM116-2a, pBTM116-Motifs, and pBTM116-GDD) consisting of
264
265 111 LexA and the RdRp-derived proteins of the CMV strain I17F (Group I) (Fig. 1) were checked for
266
267 112 correct expression in the yeast reporter strain, using Western blot and an anti-LexA antibody. The
268
269 113 LexA fusions were stably and properly expressed, except for the full-length 2a protein that could
270
271 114 not be visualised possibly for its insufficient expression level or the inability to be recognized by the
272
273 115 anti-LexA antibody (Fig. 2).

275 116 Of the three CMV RdRp-derived proteins tested, the GDD protein transactivated the two
276
277 117 Histidine and LacZ reporter genes in the pBTM116 vector (not shown) and was therefore discarded
278
279
280 118 from further analyses. In contrast, the full length 2a and the Motifs proteins interacted with 96 and
281
282 119 25 scFvs library prey constructs, respectively (Table 1), without unspecific activation of the LexA-
283
284 120 CMV antigen. Among all the colonies obtained, 77% of the constructs against the full length 2a
285
286 121 protein and 36% against the Motifs protein were confirmed positive for interaction in the β -
287
288 122 galactosidase filter assay. Four of these exhibited the strongest signal in the β -galactosidase assay
289
290 123 (Fig. 3A) and displayed a different profile in fingerprinting analysis when digested with *AvaII* (Fig.

296
297
298 124 3B). Therefore scFvF6 and scFvF71 against the full length 2a protein and scFvM52 and scFvM181
299
300
301 125 against the Motifs protein were selected for further analysis (Table 1).

302
303 126 Amino acid alignment of these scFvs showed an identity in the range of 54-59% in the light-
304
305 127 chain of the variable domain (V_L) and a higher variation in the heavy-chain of the variable domain
306
307 128 (V_H), ranging from 34% (scFvM52 vs. scFvF71) to 80% (scFvM181 vs. scFvF6) (Fig. 3C, D, E).
308
309 129 Nucleotide sequence analysis of the V region showed that the selected scFvs belong to different Ig
310
311 130 germline families (Table 1), with the only exception of two V_H regions (scFvF6 and scFvM181) that
312
313 131 belong to the same family (IGHV5), as a consequence of the highest amino acid similarity in this
314
315 132 region.

317 133 318 319 134 2.2. *Cloning of scFvs in pCAMBIA2300 for tobacco transformation*

320
321
322 135 Stable transformation of tobacco plants was achieved by cloning the four scFvs into the
323
324 136 binary vector pCAMBIA2300 and delivering the obtained constructs using *Agrobacterium*
325
326 137 *tumefaciens* and leaf explants, under kanamycin selection (Table 2). To estimate whether selected
327
328 138 scFvs conferred CMV resistance *in planta*, a total of 18 transgenic T_0 plants were regenerated, i.e.
329
330 139 eight for the Motifs construct (2 for scFvM52 and 6 for scFvM181) and ten for the full length 2a
331
332 140 construct (5 for scFvF6 and 5 for scFvF71). Out of these T_0 lines, 6 lines expressed a scFv transgene
333
334 141 of approximately 30 kDa, i.e. lines 181.4 (expressing scFvM181), 718.1, 718.3, 718.4 (scFvF71),
335
336 142 62.4, 62.8 (scFvF6) (Fig. 4), with the strongest expression occurring in the transgenic line 718.1.

338 339 143 340 341 144 2.3. *Interaction of CMV proteins and scFvs in mammalian cells*

342
343 145 In order to analyse the cellular localization of the CMV antigens used as prey, the genes
344
345 146 encoding the 2a and Motifs proteins were cloned in the pEGFP-N1 vector and expressed in
346
347 147 mammalian cells as GFP fusions. Both the 2a and Motifs proteins were found mainly located in the
348
349 148 cell cytoplasm, as determined by confocal immunofluorescence analysis (Fig. 5A), thought with a
350
351 149 different pattern. CMV-Motifs-GFP formed aggregates probably due to the overexpression of the

355
356
357 150 exogenous protein (Kopito and Sitia, 2000), while CMV-2a-GFP was uniformly distributed (Fig.
358
359 151 5A). To determine their binding activity, each scFv construct was co-transfected with EGFP fusion
360
361 152 constructs expressing the corresponding interacting CMV antigen, 2a or Motifs. Interestingly, when
362
363
364 153 scFvM52 and scFvM181 were co-expressed with the Motifs protein (Table 2) they co-localized
365
366 154 forming the same aggregates found with CMV-Motifs-EGFP alone, confirming the positive
367
368 155 interaction between the two components (Fig. 5B). Conversely, the CMV full-length 2a protein did
369
370 156 not form aggregates in the cytoplasm, making difficult to identify a clear interaction with its
371
372 157 corresponding scFvF6 and scFvF71 in mammalian cells (data not shown).
373

374 158 375 376 159 *2.4. CMV challenge of scFv transgenic plants* 377

378
379 160 Following inoculation with the FNY strain of CMV (group I), we observed that all
380
381 161 transgenic plants derived from line 718-4.2 were asymptomatic and accumulated a virus amount
382
383 162 below the threshold limits when tested by ELISA at 2 wpi (Fig. 6 and 7). Plants of lines 62-4.3,
384
385 163 181-4.5, 718-1.2, 718-6.2 showed systemic symptoms and CMV accumulation above the threshold
386
387 164 limits at 1 wpi, but when tested at 2 wpi the virus amount significantly decreased below the
388
389 165 thresholds (Fig. 6). Furthermore, symptoms did not appear on newly emerged leaves by 3 wpi (Fig.
390
391 166 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,
392
393 167 and 528-6.2 was below or equal to 20% at 2 wpi (Suppl. Table 1), considerably lower than the
394
395 168 results obtained at 1 wpi (Fig. 6).
396
397

398 169 When the same plants were challenged with the P132 isolate belonging to the CMV II
399
400 170 group, transgenic lines 181-4.5, 181-7.4, 528-4.5.6, and 718-1.2 showed CMV level below the
401
402 171 threshold limit (Fig. 7). Again, the level of CMV infection was noticeably reduced at 2 wpi
403
404 172 compared to 1 wpi, and systemic symptoms strongly decreased at 3 wpi (Fig. 7).
405
406
407 173
408

409 174 **4. Discussion** 410 411 412 413

414
415
416 175 The intrabody-based *in vivo* protein knockdown strategy has been used successfully to
417
418 176 develop plant virus resistance, as an alternative to other approaches such as RNA interference
419
420
421 177 (Jaeger et al., 2000). The choice of the target virus protein, the subcellular localization of the
422
423 178 antigen, and the level of expression of the intrabodies are the crucial points to consider to achieve
424
425 179 efficient plant virus resistance. The first reports mainly used the CP as viral target of the antibodies
426
427 180 and of scFvs (Villani et al., 2005). However, the concern that the CP diversity among plant viruses
428
429 181 would negatively influence resistance coverage led to select more efficient antibodies and scFvs
430
431 182 interacting with viral proteins with a higher level of amino acid conservation, such as the
432
433 183 polymerase enzymes. Consequently, further reports relied on the high affinity of the antibodies to
434
435 184 the polymerases of a few viruses, such as the antibody 5B-12B7 against the hepatitis C virus RdRp
436
437
438 185 (Moradpour et al., 2002), the scFv against the tomato bushy stunt virus RdRp (Boonrod et al., 2004)
439
440 186 and against the replication initiator protein of tomato yellow leaf curl virus (Safarnejad et al., 2009).

441
442 187 This work represents the first report on the use of scFvs selected against the CMV RdRp to
443
444 188 obtain CMV resistance in transgenic tobacco plants. Notably, the scFvs identified in this work were
445
446 189 initially detected for their interaction with the non-structural rotavirus protein NSP5; here, we
447
448 190 demonstrated that these scFvs were not only successfully expressed in transgenic plants, but also
449
450 191 interacted positively with the non-structural polymerase enzyme RdRp of the non-homologous virus
451
452 192 CMV. There are very limited reports on scFvs binding to antigenic determinants of non-
453
454 193 homologous proteins. A single variable domain of the shark immunoglobulin antibody was found to
455
456
457 194 specifically interact with the hepatitis B virus pre-core antigen (Walsh et al., 2011). Our results
458
459 195 demonstrate that specific interactions between the scFvs raised against an antigen of a non-
460
461 196 structural protein of a human virus are possible also with an antigen deriving from a plant virus.
462
463 197 This could be explained considering that the function of the NSP5 protein which is involved in the
464
465 198 rotavirus replicative cycle (Vascotto et al., 2004). As the NSP5 protein used in the scFv screening
466
467 199 strongly interacted with the rotavirus RdRp (VP1) (Arnoldi et al., 2007), this could also occur with
468
469
470
471
472

473
474
475 200 polymerases of other viruses, such as CMV, even if no relevant similarity was detected between
476
477 201 rotavirus VP1 and CMV 2a protein sequences (not shown).
478

479
480 202 In this study, scFvF6 and scFvF71 that specifically interacted with the CMV full-length 2a
481
482 203 protein and scFvM52 and scFvM181 that specifically bound the CMV Motifs (including the
483
484 204 conserved motifs IV-VII of this protein) were selected. Since the GDD sequence positioned within
485
486 205 the motif VI of 2a has been trans-activated, but a positive interaction was obtained with CMV
487
488 206 Motifs, this interaction could be related to the presence of a specific epitope in the IV-V or VII
489
490 207 subdomains of 2a, or to different epitopes distributed along the IV-VII domains. However, the
491
492 208 importance of the full CMV 2a protein (and the possible involvement of other epitopes) should not
493
494 209 be underestimated since the average protection level evaluated at 3 wpi against CMV was slightly
495
496 210 higher for transgenic lines expressing scFvs against 2a (89%) than against Motifs (80%).
497

498
499 211 A previous report described the selection of a scFv from the phage display F8 library that
500
501 212 specifically interacted with CMV virions in transgenic tomato plants, binding the virus in the
502
503 213 inoculated leaves and preventing systemic infection and long distance movement (Villani et al.,
504
505 214 2005). Compared to these plants, the transgenic tobacco plants of this study showed a slightly
506
507 215 higher level of CMV resistance upon infection with the same CMV strain (FNY), as only 11% of
508
509 216 the plants transformed with scFV against CMV 2a were susceptible, compared to 20% of the CMV
510
511 217 CP-scFv plants. This result might be related to the choice of the antigen (RdRp vs. CP) or to the
512
513 218 transformed host species (tobacco vs. tomato).
514

515
516 219 In spite of the difficulties to achieve scFvs accumulation in the cytosol due to the reducing
517
518 220 environmental conditions that affect antibody folding and stability (Marschall et al., 2011), we
519
520 221 could demonstrate that the intrabodies selected in this study were successfully expressed in the
521
522 222 cytoplasm in a mammalian system, and this could presumably occur also in plants. The successful
523
524 223 expression of scFv through correct antibody folding and disulfide bond-formation could be
525
526 224 mediated by endoplasmic reticulum (ER) enzymes, such as the ER protein disulfide isomerase, as
527
528 225 already reported (Ellgaard and Ruddock, 2005). Since the 2a protein is localized in the tonoplast of
529

532
533
534 226 infected tobacco cells (Cillo et al., 2002), a positive interaction of the two proteins might occur in
535
536 the cytoplasm of tobacco cells.
537 227

538
539 228 Furthermore, a relationship between CMV resistance and scFv expression was noticed. In
540
541 229 fact, all transgenic lines expressing the scFv at levels detectable by Western blot (Fig. 4) resulted
542
543 230 the most resistant against both CMV I and II groups. This highlights the potential role of correct
544
545 231 folding and expression of intrabodies in a proper cell compartment. Notably, this relationship was
546
547 232 also important in conferring resistance to other plant viruses (Xu et al., 2006; Cervera et al., 2010).
548
549 233 However, since resistance was obtained also in transgenic lines that did not show detectable levels
550
551 234 of scFv expression (particularly lines 528-4.5 and 718-6.2), it might be that only small quantities of
552
553 235 scFvs are necessary in the cytosol to induce resistance, as reported for plum pox virus resistance
554
555 236 obtained with low accumulation levels of scFvs in plants (Gil et al., 2011).
556

557
558 237 In the inoculation assays with a CMV strain belonging to group I, which represents the more
559
560 238 aggressive strains (Carrère et al., 1999), we assumed that the sufficient expression of scFv was
561
562 239 reached in transgenic line 718.4.2 to successfully bind the CMV 2a protein and therefore interfere
563
564 240 with CMV replication, conferring virus resistance and low virus titre. On the other hand, when
565
566 241 plants were challenged with CMV group II, representing mild strains, the scFvs expressed in
567
568 242 transgenic lines 718.1.2 and 181.4.5 were apparently sufficient to disarm CMV 2a and CMV-
569
570 243 Motifs, respectively resulting in a low virus titre. Interestingly, the scFvs engineered into plants
571
572 244 were initially selected against the 2a protein of CMV I subgroup, but were efficient also against a
573
574 CMV strain of another subgroup. It would be interesting to evaluate the level of resistance of these
575 245
576 plants when challenged with other cucumoviruses or with more distant members of the family
577 246
578 *Bromoviridae*.
579 247

580
581 248 The phenotype of inoculated transgenic plants differed considerably between 1 and 3 wpi. In
582
583 249 fact, all transgenic lines that were symptomatic at 1 wpi showed a decrease in CMV systemic
584
585 250 symptoms within the following weeks, until complete symptom remission in five transgenic lines
586
587 251 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
588
589
590

591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649

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’ CGGGATCCGTATGGCTTCCCTGCCCCGCATTC 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

650
651
652 278 endonuclease restriction sites (underlined) (*Bam*HI, *Pst*I, *Eco*RI, *Pst*I, *Eco*RI, and *Pst*I,
653
654 279 respectively) for cloning purposes. PCR conditions were 94°C for 5 min for denaturation, followed
655
656 280 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
657
658
659 281 min at 72°C. Bait constructs pBTM116-2a, pBTM116-Motifs, and pBTM116-GDD were obtained
660
661 282 by cloning amplicons obtained with the above primers, which resulted in fusion to the LexA
662
663 283 fragment flanked by an ADH1 transcription promoter and terminator of pBTM116 vector. Besides,
664
665 284 pBTM116-NSP5 containing the non-structural protein NSP5 of rotavirus (Vascotto et al., 2005)
666
667 285 was used as a positive control.
668

669 286 A mouse scFv library produced for the NSP5 protein *Human rotavirus A* in the prey vector
670
671 287 pVP16/D (Fig. 1C and 1D), containing 4 x 10⁴ colonies with 95% diversity (Vascotto et al., 2005)
672
673 288 was used in the Y2H system to identify positive interactions with the above CMV proteins.
674
675
676 289 Vascotto et al. (2005) reported various intrabodies that were unable to interact with the rotavirus
677
678 290 NSP5 protein in the cytoplasm of transfected mammalian cells. Because of their high diversity, it
679
680 291 was assumed that that they could be used to screen for specific interactions against other proteins.
681

682 292 683 684 685 293 4.2. *Yeast two-hybrid selection assays* 686

687 294 All Y2H assays were done as described in previously published protocols, using the
688
689 295 *Saccharomyces cerevisiae* reporter strain L40 (MATa his3–200 trp1–901 leu2–3, 112 ade2
690
691 296 LYS2::(lexAop)₄-HIS3 URA::(lexAop)₈-lacZ GAL4 gal80) (Vojtek et al., 1993). All LexA-
692
693
694 297 fusion constructs (pBTM116-2a, pBTM116-Motifs, and pBTM116-GDD) were checked by
695
696 298 Western blot for correct expression in the yeast reporter strain. For this, an overnight yeast culture
697
698 299 was diluted in YC medium (Clontech, Mountain View, CA, USA) at OD₆₀₀ =0.15 and grown at
699
700 300 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
701
702 301 pellet denatured by boiling in Laemmli buffer, resolved on 12% SDS-PAGE, and transferred onto a
703
704 302 PVDF membrane (Millipore, Burlington, MA, USA). For antigen visualization, anti-LexA
705
706
707
708

709
710
711 303 polyclonal antibody at 1:2.000 dilution (ThermoFisher Scientific, Waltham, MA, USA), followed
712
713 304 by anti-rabbit-AP antibody (ThermoFisher Scientific) were used.

715
716 305 *S. cerevisiae* L40 containing the bait plasmid (pBTM116-2a, pBTM116-Motifs, pBTM116-
717
718 306 GDD, pBTM116-NSP5) was transformed with pVP16/D-scFv and grown on selective medium,
719
720 307 followed by a β -galactosidase filter assay, as described by Visintin et al (1999). Four selected scFvs
721
722 308 which interacted with the CMV RdRp (scFvM52 and scFvM181 with CMV Motifs, and scFvF6 and
723
724 309 scFvF71 with CMV full-length 2a) were cloned into different expression vectors for further
725
726 310 investigations (pCAMBIA2300 and pEGFP-N1).

727
728 311
729
730 312 *4.3. Cloning of scFvs in pCAMBIA2300 for stable tobacco transformation*

731 313 For cloning in the binary vector pCAMBIA2300 (Cambia, Canberra, Australia), PCR was
732
733 314 performed with primers specific for the four selected scFvs, containing the SV5-tag at the 3'
734
735 315 terminus of the heavy-chain variable domains (V_H), i.e. 52_71F (5'
736
737 316 CATCGATACAATGGCCCATGCCGACATTCAGATG 3') and 52R (5'
738
739 317 CATCGATTTAGGTAGAATCGAGGCCGAGGAGAGGGTTAGGGATAGGCTTGCCTGCAGA
740
741 318 GACAGTGACCAGAGTCCCTTGGCC 3') for scFvM52, 52_71F (5'
742
743 319 CATCGATACAATGGCCCATGCCGACATTCAGATG 3') and 181R (5'
744
745 320 CATCGATTTAGGTAGAATCGAGGCCGAGGAGAGGGTTAGGGATAGGCTTGCCTGAGGA
746
747 321 GACGGTGACTGAGGTCCCTGCGCC 3') for scFvM181, 6F (5'
748
749 322 CATCGATACAATGGCCCATGCCGATATTGTAATG 3') and 6R (5'
750
751 323 CATCGATTTAGGTAGAATCGAGGCCGAGGAGAGGGTTAGGGATAGGCTTGCCTGAGGA
752
753 324 GACGGTGACCGTGGTGCCTTGGCC 3') for scFvF6, and 52_71F (5'
754
755 325 CATCGATACAATGGCCCATGCCGACATTCAGATG 3') and
756
757 326 71R(5'CATCGATTTAGGTAGAATCGAGGCCGAGGAGAGGGTTAGGGATAGGCTTGCCTG
758
759 327 AGGAGACTGTGAGAGTGGTGCCTTGGCC 3') for scFvF71.

768
769
770 328 PCR products were cloned in-frame into the *NruI* restriction site of pCAMBIA2300, modified with
771
772 329 the expression cassette of the pFF19 vector under the control of the enhanced cauliflower mosaic
773
774 virus 35S promoter. Cloned vectors were introduced into *A. tumefaciens* strain C58 by freeze/thaw
775 330
776 transformation, according to An et al., 1988.
777 331

778
779
780 332
781
782 333 *4.4. Transformation of N. tabacum*

783
784 334 Stably transformed tobacco plants (*N. tabacum* var. *Xanthi*) were generated by leaf disc
785
786 335 transformation with recombinant *A. tumefaciens*, essentially as described by Horsch et al., 1985.
787
788 336 Transgenic plants were grown in greenhouse conditions with a 16/8 h (light/dark) photoperiod.
789
790 337 Expression of scFv was determined by Western blot on total proteins extracted from transgenic
791
792 338 tissue after homogenization in Laemmli sample buffer (approximately 9 µl/mg tissue). The western
793
794 339 blot was developed using an anti-SV5 MAb diluted at 1:5000, followed by anti-mouse HRP Ab
795
796 (KPL, SeraCare, Milford, MA, USA).
797 340

798
799 341
800
801 342 *4.5. Cloning of CMV genes into pEGFP-N1 and scFvs into pcDNA3 and their expression in*
802
803 343 *mammalian cells*

804
805 344 cDNA sequences encoding the CMV 2a and Motifs proteins were amplified using the
806
807 345 following primers: 409for (5' CGCTGCAGGCCACCATGGCTTTCCTGCCCCCGCATTC 3')
808
809 346 and 410rev (5'**CGGGATCC**GACTCGGGTAACTCCGCCACGTTC 3') for 2a, and 411for
810
811 347 (5'**CGGAATTCTGGCCACCATGGATCTGTCTAAGTTTGATAAGTCTC** 3') and 253rev (5'
812
813 CGCTGCAGCTTCGAACAAATATATGGTACGGCA 3') for Motifs. PCR CMV 2a and Motifs
814 348
815 products were cloned into *PstI* (underlined) and *BamHI* (bold) of pEGFP-N1 (Clontech) vector,
816 349
817 fused in frame with the enhanced green fluorescent protein (EGFP) gene.
818 350

819
820 351 scFvs in pVP16/D were digested with *NheI* and *HindIII* restriction enzymes and cloned into the
821
822 352 same restriction fragment of the pcDNA3 vector (ThermoFisher Scientific). pcDNA3-M52,
823
824

827
828
829 353 pcDNA3-M181, pcDNA3-F6, and pcDNA3-F71 constructs were tagged with two nuclear
830
831 354 localization signals (NLS) of SV40 T-antigen within the *NheI* restriction site using annealed
832
833
834 355 oligonucleotides, as described by Vascotto *et al.* (2005).
835
836 356

837
838 357 *4.6. Mammalian cells culture and transfection*
839

840 358 Rhesus monkey kidney MA104 cells were routinely cultured in Dulbecco's modified
841
842 359 Eagle's medium containing 10% foetal calf serum (Gibco, ThermoFisher Scientific). Cell cultures
843
844 360 maintained in the absence of serum and antibiotics (serum-free medium) were used for DNA
845
846 361 transfections. Transient transfections with vaccinia virus were performed as previously described
847
848
849 362 (Eichwald *et al.*, 2002). One tenth of the volume of total cellular extracts was used in Western blot
850
851 363 analysis. For cells transfected with scFvs, an anti-SV5 MAb diluted at 1:5000, followed by anti-
852
853 364 mouse HRP Ab (KPL) were used, while for cells transfected with CMV 2a and Motifs, an anti-GFP
854
855 365 rabbit polyclonal Ab (ThermoFisher Scientific) diluted at 1:2000, followed by anti-rabbit HRP Ab
856
857 366 (ThermoFisher Scientific) were used.
858

859 367
860
861 368 *4.7. Immunofluorescence microscopy*
862

863 369 Transfected cells were washed twice with phosphate buffered saline (PBS) and fixed with
864
865 370 3.7% paraformaldehyde for 10 min at room temperature. After fixation, the cells were washed three
866
867
868 371 times with PBS and permeabilised with 0.1% Triton in PBS for 5 min. Next, samples were washed
869
870 372 with PBS, and non-specific binding sites were blocked with 1% BSA in PBS for 30 min. Slides
871
872 373 were incubated with anti-SV5 MAb and anti-GFP Ab for 1 h in a moist chamber. Anti-mouse
873
874 374 RITC-conjugated antibody (Pierce, Rockford, IL, USA) was used at a 1:600 dilution. Thereafter,
875
876 375 samples were washed and mounted using ProLong mounting medium (Molecular Probes, Eugene,
877
878 376 OR, USA). Images were acquired with an argon-helium double laser confocal microscope (Zeiss,
879
880 377 Oberkochen, Germany).
881

882 378
883
884
885

886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944

379 4.8. *Virus challenge of scFv transgenic tobacco plants*

380 T2 tobacco plants were grown in soil and maintained in greenhouse conditions at 20–28/16–
381 20°C (day/night), with and a 16/8 h (light/dark) photoperiod. Individual plantlets with three fully
382 developed leaves were dusted with abrasive powder (Carborundum, Sigma, Kawasaki, Japan) and
383 inoculated by rubbing the upper surface with fingers dipped in the virus inoculum. This consisted of
384 extracts from young symptomatic leaves of *N. benthamiana* infected by the CMV FNY or P132
385 isolates, or of a healthy plant, ground in ice-cold inoculation buffer (10 mM phosphate buffer, pH
386 7.0, 5 mM Na-diethyldithiocarbamate, 1 mM EDTA, and 5 mM thioglycolic acid-Na salt). Non-
387 transformed tobacco plants var. Xanthi were used as positive control plants. Inoculated plants (n=6)
388 were maintained for up to 6 weeks post-inoculation for symptom scoring.

390 4.9. *Double-Antibody Sandwich/Enzyme linked immunosorbent assay (DAS-ELISA)*

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

398 5.0. *Statistical analysis*

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

404 5.1. **Conclusion**

945
946
947 405 In conclusion, from an NSP5 scFv library, we selected four intrabodies that positively
948
949 406 interacted with the full-length 2a and the 2a conserved motif region of the CMV polymerase.
950
951
952 407 Following their transformation in tobacco, we observed that they conferred resistance to both CMV
953
954 408 subgroups I and II. Higher resistance was achieved with intrabodies targeting the full-length 2a
955
956 409 protein, highlighting the possibility that more than one epitope is involved in the positive protein
957
958 410 interactions required to confer CMV resistance.

960 411 **Acknowledgement**

962 412
963
964 413 The authors wish to thank Dr. Oscar Burrone (International Centre for Genetic Engineering
965
966 414 and Biotechnology, Trieste, Italy) for initial discussions and providing the original scFv library. We
967
968
969 415 are grateful to Dr. Marina Ciuffo (IPSP-CNR) for help with DAS-ELISA, to Elena Zocca (IPSP-
970
971 416 CNR) and Mirco Boem (ICGEB Biosafety Outstation) for plant care, and Laura Chiappetta (ICGEB
972
973 417 Biosafety Outstation) for technical support.

974 975 418 **Competing interests**

976
977 419
978
979 420 The authors declare they have no competing interests.
980
981 421

982 983 422 **References**

- 984
985 423 Aebig, J.A., Albert, H.H., Zhu, B.L., Hu, J.S., Hsu, H.T., 2006. Cloning and construction of single-
986
987 chain variable fragments (scFv) to Cucumber mosaic virus and production of transgenic
988 424 plants. *Acta Hortic.* 722, 129–136.
989
990 425
991
992 426 Ahmad, Z.A., Yeap, S.K., Ali, A.M., Ho, W.Y., Alitheen, N.B.M., Hamid, M., 2012. scFv
993
994 427 antibody: principles and clinical application. *Clin. Dev. Immunol.* 2012, 980250.
995
996
997 428 An, G., Ebert, P.R., Mitra, A., Ha S.B., 1988. Binary vectors, in: Gelvin, S.B., Schilperoort, R.A.
998
999 429 (Eds.), *Plant Molecular Biology Manual*, vol. A3. Kluwer Academic Publishers, Dordrecht,
1000
1001
1002
1003

1004
1005
1006 430 pp. 1–19.
1007
1008
1009 431 Arnoldi, F., Campagna, M., Eichwald, C., Desselberger, U., Burrone, O.R., 2007. Interaction of
1010
1011 432 rotavirus polymerase VP1 with nonstructural protein NSP5 is stronger than that with NSP2. J.
1012
1013 433 Virol. 81, 2128–37.
1014
1015
1016 434 Ayadi, M., Bouaziz, D., Nouri-Ellouz, O., Rouis, S., Drira, N., Gargouri-Bouزيد, R., 2012. Efficient
1017
1018 435 resistance to *Potato virus Y* infection conferred by cytosolic expression of anti-viral protease
1019
1020 436 single-chain variable fragment antibody in transgenic potato plants. J Plant Pathol. 94, 561–
1021
1022 437 569.
1023
1024
1025 438 Boonrod, K., Galetzka, D., Nagy, P.D., Conrad, U., Krczal, G., 2004. Single-chain antibodies
1026
1027 439 against a plant viral RNA-dependent RNA polymerase confer virus resistance. Nat.
1028
1029 440 Biotechnol. 22, 856–862.
1030
1031
1032 441 Carrère, I., Tepfer, M., Jacquemond, M., 1999. Recombinants of cucumber mosaic virus (CMV):
1033
1034 442 determinants of host range and symptomatology. Arch. Virol. 144, 365–379.
1035
1036
1037 443 Cervera, M., Esteban, O., Gil, M., Gorrís, M.T., Martínez, M.C., Peña, L., Cambra, M., 2010.
1038
1039 444 Transgenic expression in citrus of single-chain antibody fragments specific to *Citrus tristeza*
1040
1041 445 *virus* confers virus resistance. Transgenic Res. 19, 1001–1015.
1042
1043
1044 446 Cillo, F., Roberts, I.M., Palukaitis, P., 2002. In situ localization and tissue distribution of the
1045
1046 447 replication-associated proteins of *Cucumber mosaic virus* in tobacco and cucumber. J. Virol.
1047
1048 448 76, 10654–10664.
1049
1050 449 Chapman, S., Kavanagh, T., Baulcombe, D.C., 1992. Potato virus X as a vector for gene expression
1051
1052 450 in plants. Plant J. 2, 549–557.
1053
1054
1055 451 Choi, S., Lee, J.H., Kang, W.H., Kim, J., Huy, H.N., Park, S.W., Son, E.H., Kwon, J.K., Kang,
1056
1057 452 B.C., 2018. Identification of *Cucumber mosaic resistance 2 (cmr2)* that confers resistance to a
1058
1059 453 new *Cucumber mosaic virus* isolate P1 (CMV-P1) in pepper (*Capsicum* spp.). Front Plant Sci.
1060
1061
1062

1063
1064
1065 454 9:1106.
1066
1067
1068 455 Clark, M.F., Adams, A.N., 1977. Characteristics of microplate method of enzyme linked
1069
1070 456 immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 34, 475–483.
1071
1072
1073 457 Crivianu-Gaita, V., Thompson, M., 2016. Aptamers, antibody scFv, and antibody Fab' fragments:
1074
1075 458 An overview and comparison of three of the most versatile biosensor biorecognition elements.
1076
1077 459 *Biosens. Bioelectron.* 85, 32–45.
1078
1079
1080 460 De Jaeger, G., De Wilde, C., Eeckhout, D., Fiers, E., Depicker, A., 2000. The plantibody approach:
1081
1082 461 expression of antibody genes in plants to modulate plant metabolism or to obtain pathogen
1083
1084 462 resistance. *Plant Mol. Biol.* 43, 419–428.
1085
1086
1087 463 Edwardson, J.R., Christie R.G., 1991. Cucumoviruses, *CRC Handbook of viruses infecting*
1088
1089 464 legumes. CRC Press, Boca Raton.
1090
1091
1092 465 Eichwald, C., Vascotto, F., Fabbretti, E., Burrone, O.R., 2002. Rotavirus NSP5: mapping
1093
1094 466 phosphorylation sites and kinase activation and viroplasm localization domains. *J. Virol.* 76,
1095
1096 467 3461–3470.
1097
1098
1099 468 Ellgaard, L., Ruddock, L.W., 2005. The human protein disulphide isomerase family: substrate
1100
1101 469 interactions and functional properties. *EMBO Rep.* 6, 28–32.
1102
1103
1104 470 Gargouri-Bouزيد, R., Jaoua, L., Rouis, S., Saïdi, M.N., Bouaziz, D., Ellouz, R., 2006. PVY-resistant
1105
1106 471 transgenic potato plants expressing an anti-NIa protein scFv antibody. *Mol Biotechnol.* 33:
1107
1108 472 133–140.
1109
1110
1111 473 Gil, M., Esteban, O., García, J.A., Peña, L., Cambra, M., 2011. Resistance to *Plum pox virus* in
1112
1113 474 plants expressing cytosolic and nuclear single-chain antibodies against the viral RNA N1b
1114
1115 475 replicase. *Plant Pathol.* 60, 967–976.
1116
1117
1118 476 Hayes, R.J., Buck, K.W., 1990. Complete replication of a eukaryotic virus RNA in vitro by a
1119
1120 477 purified RNA-dependent RNA polymerase. *Cell* 63, 363–368.
1121

1122
1123
1124
1125 478 Horsch, R.B., Fry, J.E., Hoffman, N.L., Rogers, S.G., Fraley, R.T., 1985. A simple and general
1126
1127 479 method for transferring genes into plants. *Science* 227, 1229–1231.
1128
1129 480 Jacquemond, M., 2012. Cucumber mosaic virus. *Adv. Virus Res.* 84, 439–504.
1130
1131
1132 481 Koonin, E.V., 1991. The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA
1133
1134 482 viruses. *J. Gen. Virol.* 72, 2197–2206.
1135
1136
1137 483 Kopito, R. R., Sitia, R., 2000. Aggresomes and Russell bodies. *EMBO Rep.* 1, 225–231.
1138
1139 484 Liu, H.L., Lin, W.F., Hu, W.C., Lee, Y.A., Chang, Y.C., 2015. A Strategy for Generating a Broad-
1140
1141 485 Spectrum Monoclonal Antibody and Soluble Single-Chain Variable Fragments against Plant
1142
1143 486 Potyviruses. *Appl. Environ. Microbiol.* 81, 6839–6849.
1144
1145
1146 487 Marschall, A.L.J., Frenzel, A., Schirrmann, T., Schüngel, M., Dübel, S., 2011. Targeting antibodies
1147
1148 488 to the cytoplasm. *mAbs* 3, 3–16.
1149
1150
1151 489 Moradpour, D., Bieck, E., Hügler, T., Wels, W., Wu, J.Z., Hong, Z., Blum, H.E., Bartenschlager, R.,
1152
1153 490 2002. Functional properties of a monoclonal antibody inhibiting the hepatitis C virus RNA-
1154
1155 491 dependent RNA polymerase. *J. Biol. Chem.* 277, 593–601.
1156
1157
1158 492 Morroni, M., Thompson, J.R., Tepfer, M., 2008. Twenty years of transgenic plants resistant to
1159
1160 493 Cucumber mosaic virus. *Mol. Plant Microbe Interact.* 21, 675–684.
1161
1162 494 Nickel, H., Kawchuk, L., Twyman, R.M., Zimmermann, S., Junghans, H., Winter, S., Fischer, R.,
1163
1164 495 Prüfer, D., 2008. Plantibody-mediated inhibition of the *Potato leafroll virus* P1 protein
1165
1166 496 reduces virus accumulation. *Virus Res.* 136, 140–145.
1167
1168
1169 497 O'Reilly, E.K., Kao, C.C., 1998. Analysis of RNA-dependent RNA polymerase structure and
1170
1171 498 function as guided by known polymerase structures and computer predictions of secondary
1172
1173 499 structure. *Virology* 252, 287–303.
1174
1175
1176 500 O'Reilly, E.K., Wang, Z., French, R., Kao, C.C., 1998. Interactions between the structural domains
1177
1178 501 of the RNA replication proteins of plant-infecting RNA viruses. *J. Virol.* 72, 7160–7169.
1179
1180

1181
1182
1183
1184 502 Owen, J., Palukaitis, P., 1988. Characterization of cucumber mosaic virus. I. Molecular
1185 heterogeneity mapping of RNA 3 in eight CMV strains. *Virology* 166, 495–502.
1186 503
1187
1188 504 Palukaitis, P., García-Arenal, F., 2003. Cucumoviruses. *Adv. Virus Res.* 62, 241–323.
1189
1190
1191 505 Peschen, D., Schillberg, S., Fischer, R., 2016. Antibody-Mediated Pathogen Resistance in Plants.
1192 *Methods Mol. Biol.* 1385, 273–291.
1193 506
1194
1195 507 Prins, M., Laimer, M., Noris, E., Schubert, J., Wassenegger, M., Tepfer, M., 2008. Strategies for
1196 antiviral resistance in transgenic plants. *Mol. Plant Pathol.* 9, 73–83.
1197 508
1198
1199
1200 509 Raag, R., Whitlow, M., 1995. Single-chain Fvs. *FASEB J.* 9, 73–80.
1201
1202
1203 510 Roossinck, M.J., Bujarski, J., Ding, S.W., Hajimorad, R., Hanada, K., Scott, S., Tousignant, M.,
1204 1999. Family *Bromoviridae*, in: vanRegenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L.
1205 511 (Eds.), *Virus Taxonomy-Seventh Report of the International Committee on Taxonomy of*
1206 *Viruses*. Academic Press, San Diego, pp. 923–935.
1207 512
1208
1209 513
1210
1211
1212 514 Roossinck, M.J., 2002. Evolutionary History of *Cucumber Mosaic Virus* Deduced by Phylogenetic
1213 Analyses. *J. Virol.* 76, 3382–3387.
1214 515
1215
1216 516 Safarnejad, M.R., Fischer, R., Commandeur, U., 2009. Recombinant-antibody-mediated resistance
1217 against *Tomato yellow leaf curl virus* in *Nicotiana benthamiana*. *Arch. Virol.* 154, 457–467.
1218 517
1219
1220
1221 518 Safarnejad, M.R., Jouzani, G.S., Tabatabaei, M., Twyman, R.M., Schillberg, S., 2011. Antibody-
1222 mediated resistance against plant pathogens. *Biotechnol. Adv.* 29, 961–971.
1223 519
1224
1225
1226 520 Safarnejad, M.R., Safarpour, H., Shahryari, F., Basirat, M., Tabatabaei, M., Kordenaeej, A., Naji,
1227 A.M., Kakouienejad, M., 2013. Selection of specific single chain variable fragments (scFv)
1228 521 against *Polymyxa betae* from phage display libraries. *J. Plant Prot. Res.* 53, 357–363.
1229 522
1230
1231
1232 523 Shilpa, B.S., 2013. Development of single chain fragment variable monoclonal antibody against
1233 *Banana bunchy top virus* coat protein. MSc Thesis. Dharwad University of agricultural
1234 524 sciences.
1235
1236
1237 525
1238
1239

1240
1241
1242
1243 526 Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A., Galeffi, P., 1993.
1244
1245 527 Transgenic plants expressing a functional single-chain Fv antibody are specifically protected
1246
1247 528 from virus attack. *Nature* 366, 469–72.
1248
1249 529 Thompson, J.R., Tepfer, M., 2010. Assessment of the benefits and risks for engineered virus
1250
1251 530 resistance. *Adv. Virus Res.* 76, 33–56.
1252
1253
1254 531 Vascotto, F., Campagna, M., Visintin, M., Cattaneo, A., Burrone, O.R., 2004. Effects of intrabodies
1255
1256 532 specific for rotavirus NSP5 during the virus replicative cycle. *J. Gen. Virol.* 85, 3285–3290.
1257
1258
1259 533 Vascotto, F., Visintin, M., Cattaneo, A., Burrone, O.R., 2005. Design and selection of an intrabody
1260
1261 534 library produced de-novo for the non-structural protein NSP5 of rotavirus. *J. Immunol.*
1262
1263 535 *Methods* 301, 31–40.
1264
1265
1266 536 Villani, M.E., Roggero, P., Bitti, O., Benvenuto, E., Franconi, R., 2005. Immunomodulation of
1267
1268 537 cucumber mosaic virus infection by intrabodies selected in vitro from a stable single-
1269
1270 538 framework phage display library. *Plant Mol. Biol.* 58, 305–316.
1271
1272
1273 539 Visintin, M., Tse, E., Axelson, H., Rabbitts, T.H., Cattaneo, A., 1999. Selection of antibodies for
1274
1275 540 intracellular function using a two-hybrid in vivo system. *Proc. Natl. Acad. Sci. U. S. A.* 96,
1276
1277 541 11723.
1278
1279
1280 542 Visintin, M., Quondam, M., Cattaneo, A., 2004. The intracellular antibody capture technology:
1281
1282 543 towards the high-throughput selection of functional intracellular antibodies for target
1283
1284 544 validation. *Methods* 34, 200–214.
1285
1286
1287 545 Vojtek, A.B., Hollenberg, S.M., Cooper, J.A., 1993. Mammalian Ras interacts directly with the
1288
1289 546 serine/threonine kinase Raf. *Cell* 74: 205–214.
1290
1291
1292 547 Zhang, X.P., Liu, D.S., Yan, T., Fang, X.D., Dong, K., Xu, J., Wang, Y., Yu, J.L., Wang, X.B.,
1293
1294 548 2017. Cucumber mosaic virus coat protein modulates the accumulation of 2b protein and
1295
1296 549 antiviral silencing that causes symptom recovery *in planta*. *PLoS Pathog.* 13: e1006522.
1297
1298

1299
1300
1301
1302
1303
1304
1305
1306
1307
1308
1309
1310
1311
1312
1313
1314
1315
1316
1317
1318
1319
1320
1321
1322
1323
1324
1325
1326
1327
1328
1329
1330
1331
1332
1333
1334
1335
1336
1337
1338
1339
1340
1341
1342
1343
1344
1345
1346
1347
1348
1349
1350
1351
1352
1353
1354
1355
1356
1357

550 Zhao, M.A., An, S.J., Lee, S.C., Kim D.S., Kang, B.C., 2013. Overexpression of a single-chain
551 variable fragment (*scFv*) antibody confers unstable resistance to TuMV in Chinese cabbage.
552 Plant Mol. Biol. Rep. 31, 1203–1211.

553 Zitter, T.A., Murphy, J.F., 2009. Cucumber mosaic. The Plant Health Instructor. doi: 10.1094/PHI-
554 I-2009-0518-01

555 Xu, M.Q., Li, H.P., Wang, M., Wu, Z.C., Borth, W.B., Hsu, H.T., Hu, J.S., 2006. Transgenic plants
556 expressing a single-chain fv antibody to *Tomato spotted wilt virus* (TSWV) are resistant to
557 TSWV systemic infection. Acta Hortic. 722, 337–348.

1358
1359
1360
1361
1362
1363
1364
1365
1366
1367
1368
1369
1370
1371
1372
1373
1374
1375
1376
1377
1378
1379
1380
1381
1382
1383
1384
1385
1386
1387
1388
1389
1390
1391
1392
1393
1394
1395
1396
1397
1398
1399
1400
1401
1402
1403
1404
1405
1406
1407
1408
1409
1410
1411
1412
1413
1414
1415
1416

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.

1476
1477
1478
1479
1480
1481
1482
1483
1484
1485
1486
1487
1488
1489
1490
1491
1492
1493
1494
1495
1496
1497
1498
1499
1500
1501
1502
1503
1504
1505
1506
1507
1508
1509
1510
1511
1512
1513
1514
1515
1516

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 <i>AvaII</i> 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
Motif	25	9	80%	2	F71	KV14-111	HV8-12
					M52	KV6-25	HV1-47
					M181	KV3-12	HV5-17

1517
 1518
 1519
 1520
 1521
 1522
 1523
 1524
 1525
 1526
 1527
 1528
 1529
 1530
 1531
 1532
 1533
 1534
 1535
 1536
 1537
 1538
 1539
 1540
 1541
 1542
 1543
 1544
 1545
 1546
 1547
 1548
 1549
 1550
 1551
 1552
 1553
 1554
 1555
 1556
 1557

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 T₀ 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)

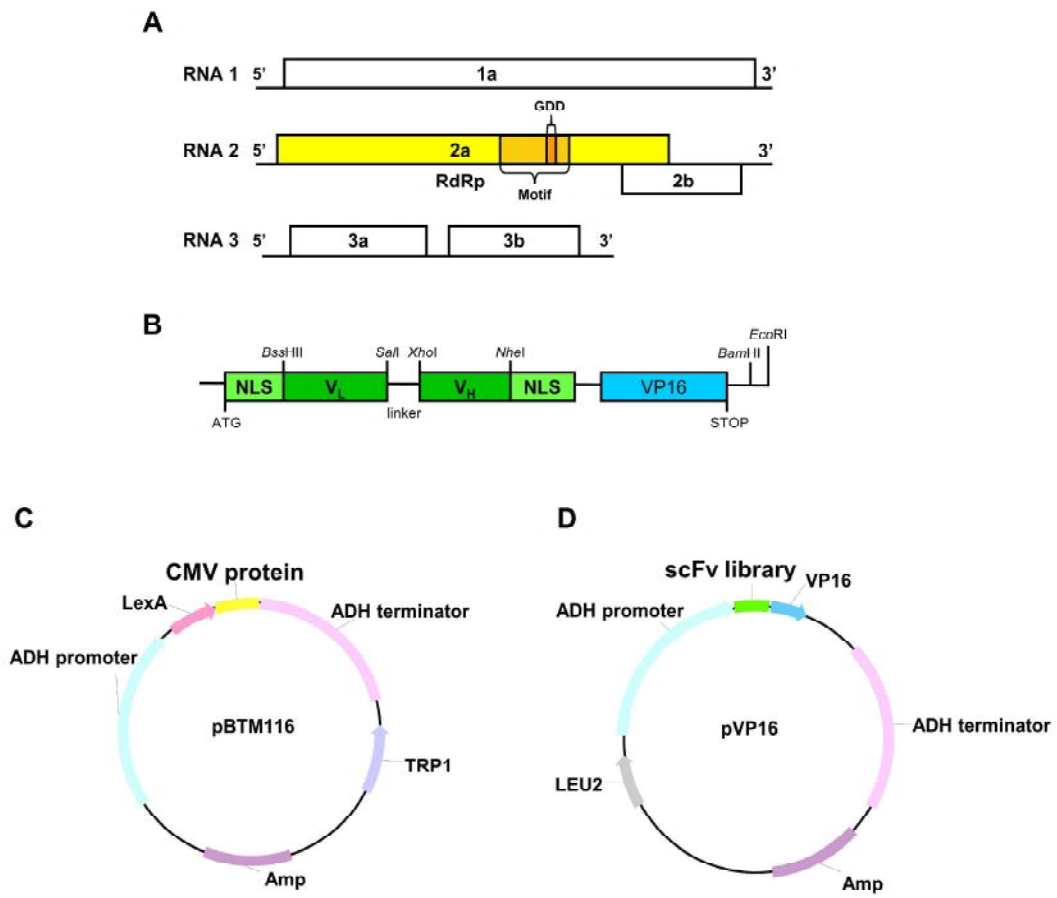


Figure 1
Matić et al.

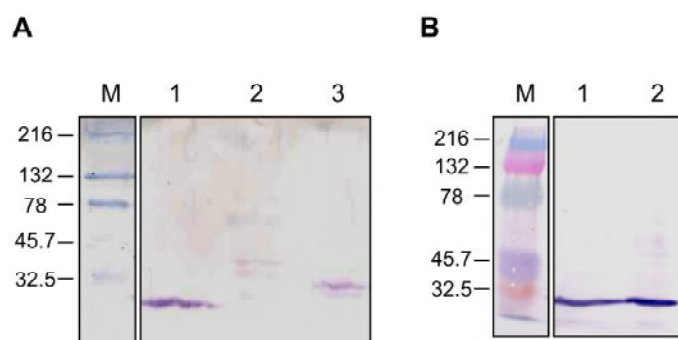


Figure 2
Matić et al.

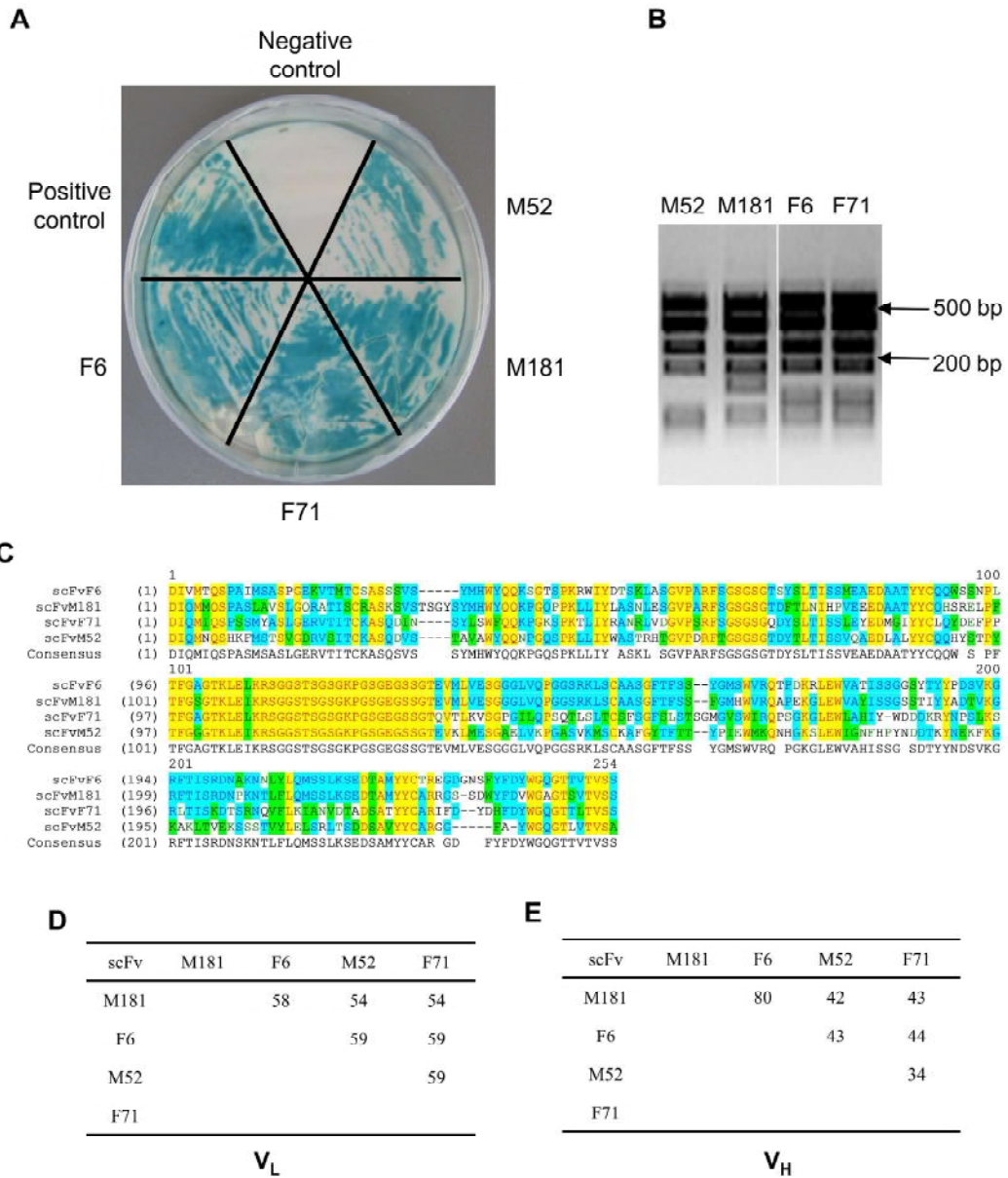


Figure 3
Matić et al.

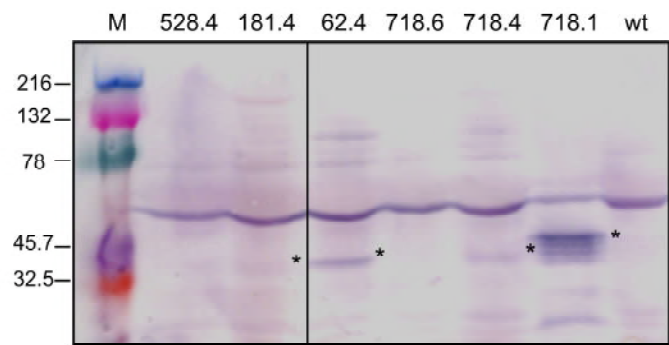


Figure 4
Matić et al.

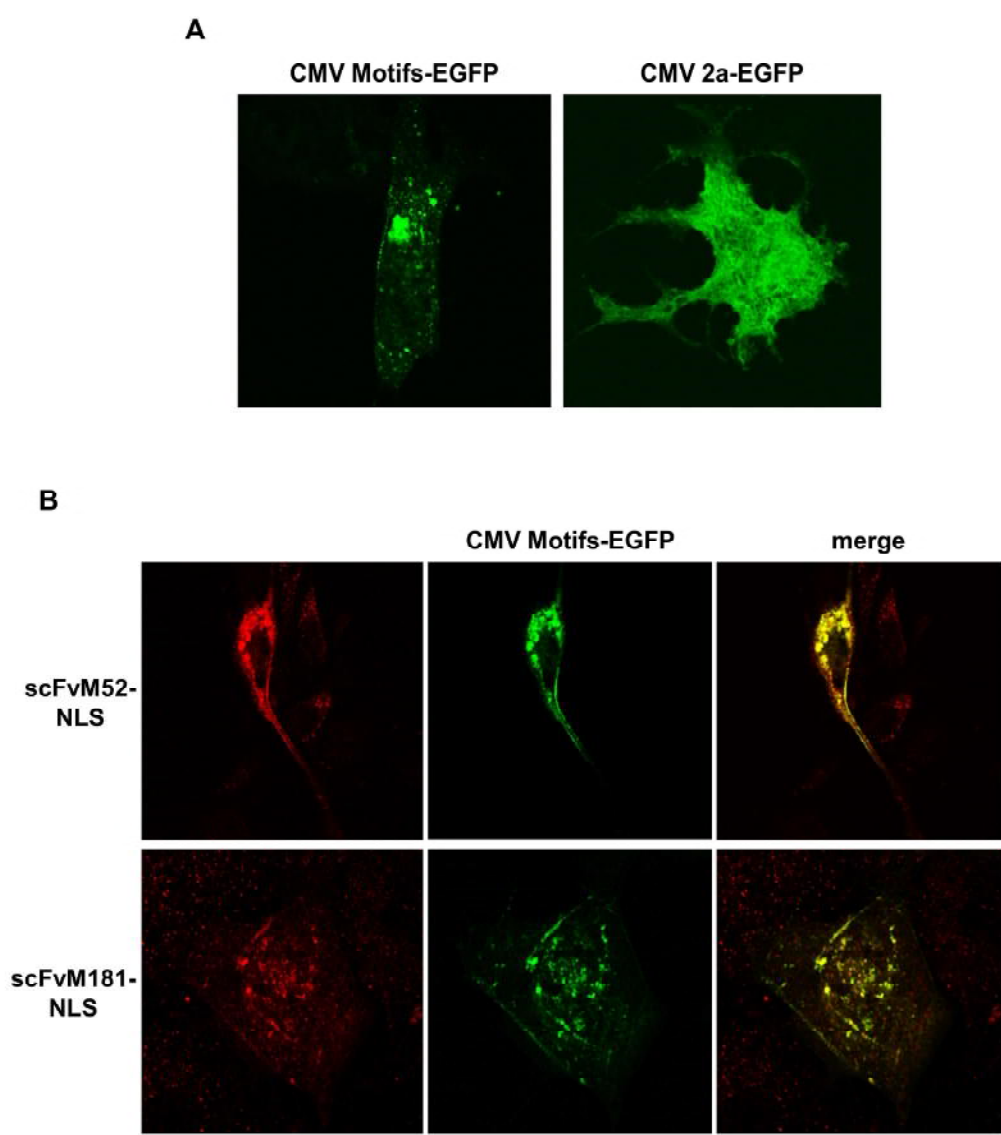


Figure 5
Matić et al.

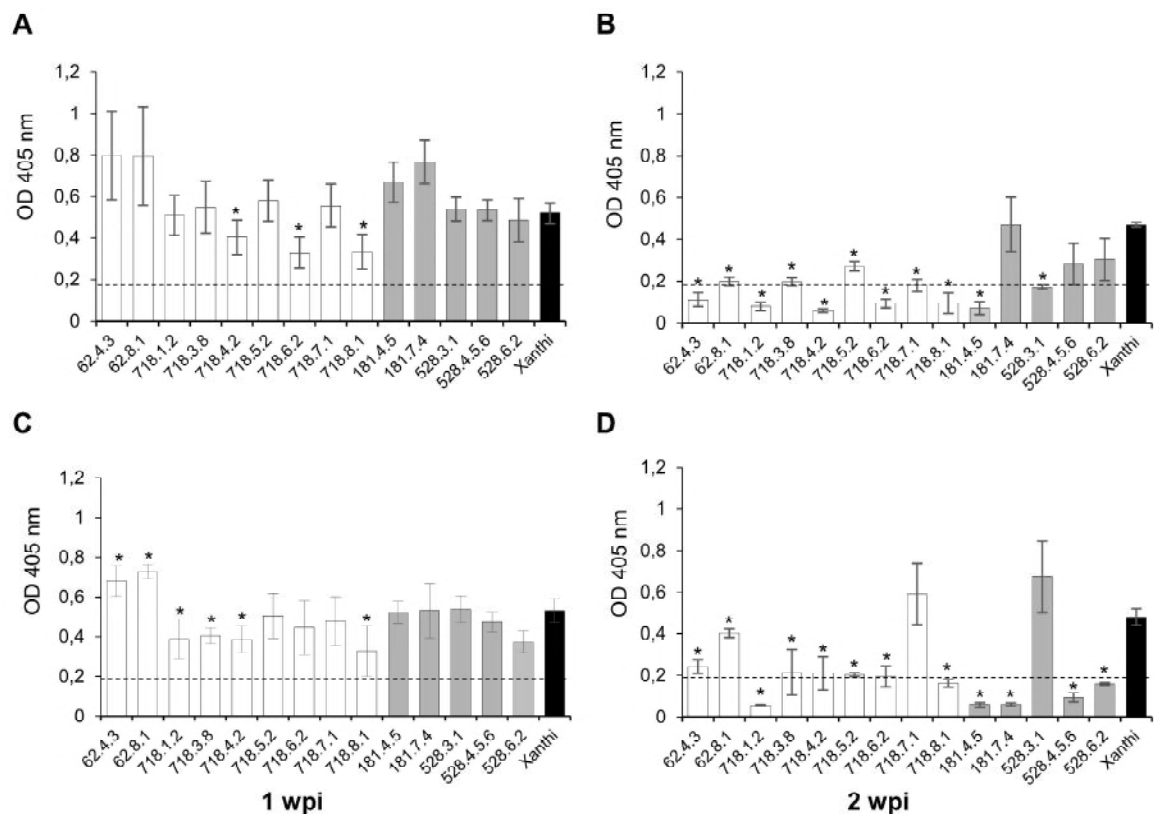


Figure 6
Matić et al.

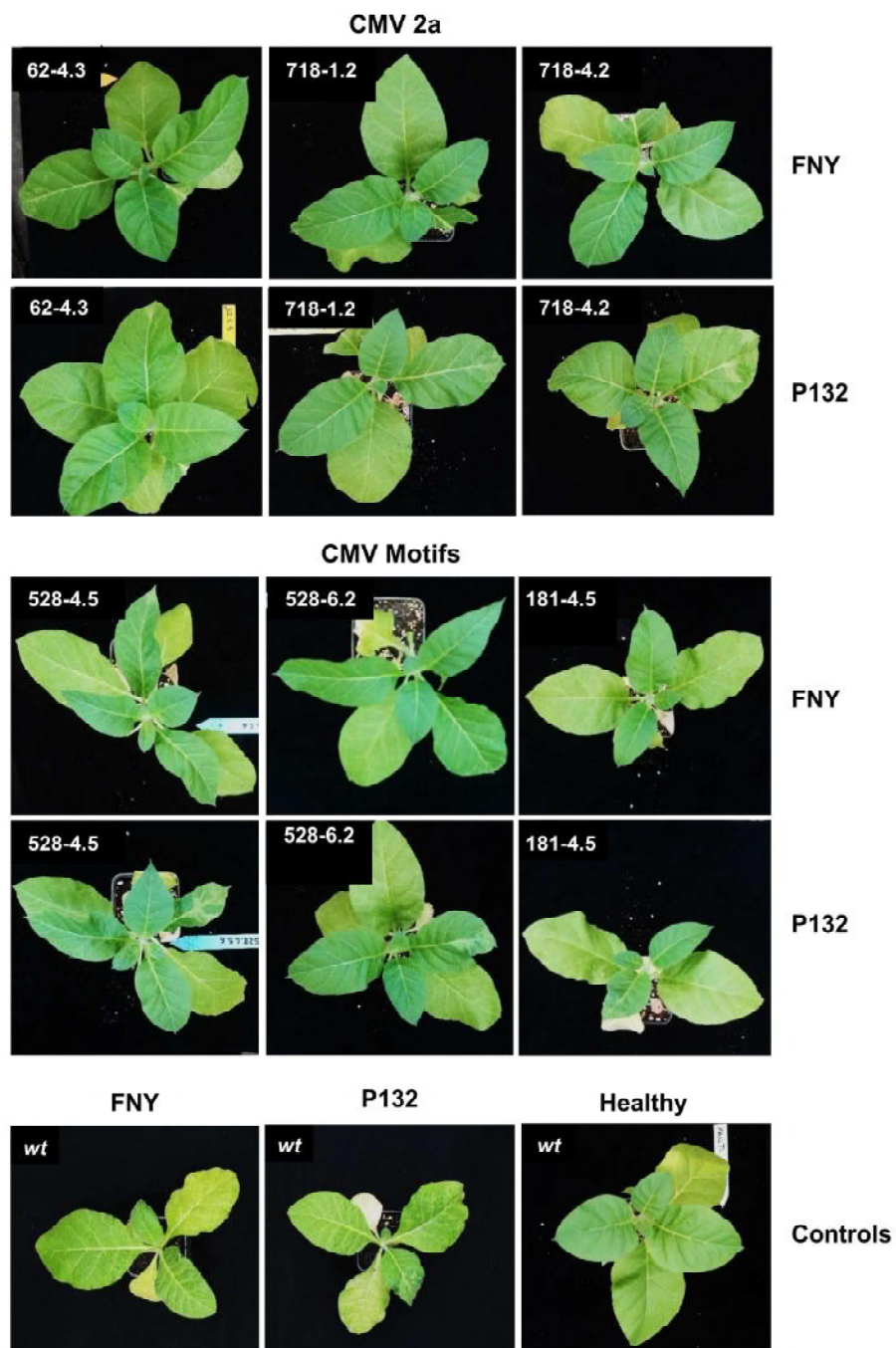


Figure 7
Matić et al.