A plant-specific syntaxin-6 protein contributes to the intracytoplasmic route for begomoviruses

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Running title: NISP in begomovirus intracellular transport

Abstract

Due to limited free diffusion in the cytoplasm, viruses must use active transport mechanisms to move intracellularly. Nevertheless, how the plant ssDNA begomoviruses hijacks the host intracytoplasmic transport machinery to move from the nucleus to the plasmodesmata remains enigmatic. Here, we identified nuclear shuttle protein (NSP)-interacting proteins from Arabidopsis by probing a protein microarray and demonstrated that the *Cabbage leaf curl virus* (CabLCV) NSP, a facilitator of the nucleocytoplasmic trafficking of viral (v)DNA, interacts with an endosomal vesicle-localized plant-specific syntaxin-6 protein, designated NSP-interacting syntaxin-6 domain-containing protein (NISP) *in planta*. NISP displays a pro-viral function, but not the syntaxin-6 paralog AT2G18860 that failed to interact with NSP. Consistent with these findings, *nisp-1* mutant plants were less susceptible to begomovirus infection, a phenotype reversed by NISP complementation. NISP-overexpressing lines accumulated higher levels of viral DNA than wild-type. Furthermore, NISP interacted with NIG, an NSP-interacting GTPase involved in NSP-vDNA nucleocytoplasmic translocation. The NISP-NIG interaction was enhanced by NSP. We also showed that NISP associates with vDNA and might assemble a NISP-NIG-NSP-vDNA- complex. NISP may function as a docking site for recruiting NIG and NSP into endosomes, providing a mechanism for the intracytoplasmic translocation of the NSP-vDNA complex towards to and from the cell periphery.

Author Summary

As viruses must use an active and directed intracellular movement, they hijack the intracellular host transport system for their own benefits. Therefore, the identification of interactions between

host proteins and begomovirus movement proteins should target the intracellular transport machinery. This work focused on the identification of these protein-protein interactions; it addressed the molecular bases for the intracellular transport of begomoviruses. We used a protein microarray to identify cellular partners for the movement protein (MP) and the viral nuclear shuttle protein (NSP), which is a facilitator of the nucleocytoplasmic trafficking of viral (v)DNA. We identified relevant protein-protein interaction (PPI) hubs connecting host and viral proteins. We revealed a novel NSP-interacting protein, which functions in the intracytoplasmic transport of proteins and DNA from begomoviruses and was designated NSP-interacting syntaxin domain-containing protein (NISP). Our data suggest an intracellular route connecting the release of newly synthesized begomoviral DNA in the cytosol with the cell surface. Resolving viral DNA-host protein complexes led to the identification of a novel class of components of the cell machinery and a representative member, NISP, that functions as a susceptibility gene against begomoviruses. As geminiviruses pose a severe threat to agriculture and food security, this recessive gene can now be exploited as a target for engineering resistance by gene editing in crops.

63 ## Introduction

64

65 As obligate intracellular parasites, viruses must enter the host cells and hijack the cell
66 machinery to complete their life cycle. Independent on the molecular mechanisms for virus entry
67 into cells, which differ entirely between animal and plant viruses, once in the cytoplasm, both plant
68 and animal viral genomes move intracellularly to specific cellular sub-compartments for
69 replication, transcription or encapsidation [1,2]. Due to the limitation of free diffusion in the
70 cytoplasm, viruses have invoked the endogenous host movement mechanisms for an active and
71 directed intracytoplasmic translocation. In fact, almost all viruses are capable of subverting the
72 microtubule transport system to some extent to replicate and spread. Plant viruses must also use
73 an active mechanism to move the viral genome from the replication site to the plasmodesmata,
74 which are intercellular cytoplasmic bridges between plant cells [2,3]. To promote this typical cell-
75 to-cell movement, plant viruses have evolved movement proteins that increase the size exclusion
76 limit of the plasmodesmata and actively promote the viral nucleic acid translocation into
77 uninfected, adjacent cells [4]. In the case of viruses that replicate in the nuclei of infected cells,
78 they have to be directed to the nuclear periphery, followed by nuclear entry. Likewise, the newly-
79 synthesized viral proteins and viral genomes must translocate throughout the cytoplasm during
80 exit. In animal cells, the retroviruses and DNA viruses deploy the microtubule motor system and
81 microtubule cytoskeleton to move viral proteins and genomes into the nucleus and back to the
82 plasma membrane [1, 5]. Geminiviruses are typical examples of plant DNA viruses that replicate
83 in the nuclei of infected cells, but the underlying mechanisms for the intracytoplasmic movement
84 of viral proteins and viral DNA (vDNA) are far less understood.

85 Geminiviruses are circular single-stranded (ss) DNA viruses, which replicate via double-

86 stranded (ds) DNA intermediates in the nucleus of infected cells, infect a broad spectrum of
87 economically important crops and cause significant yield losses worldwide [6]. The *Geminiviridae*
88 family includes nine genera, but approximately 75% of the identified species belong to the genus
89 *Begomovirus*, which are whitefly-transmitted, infect dicotyledonous species, and display a genome
90 configuration that can be either monopartite or bipartite [7,8]. The genomic components of the
91 bipartite begomoviruses are designated DNA-A and DNA-B. The DNA-A encodes proteins
92 required for viral replication (AC1 or Rep, REn or AC3), transactivation of viral genes (AC2 or
93 TRaP), encapsidation of viral genome (AV1 or CP) and suppression of host defenses (AC4 and
94 TrAP). DNA-B encodes the nuclear shuttle protein (NSP or BV1) and the movement protein (MP
95 or BC1) involved in intracellular and cell-to-cell movement of vDNA.

96 Although the role of MP-NSP complex formation in mediating intracellular and
97 intercellular transport of begomoviruses is widely accepted, the molecular mechanisms underlying
98 the action of these viral proteins at individual stages of the intracellular and intercellular movement
99 are still debated. Some studies indicated that NSP from *Bean dwarf mosaic virus* (BDMV) and
100 *Abutilon mosaic virus* (AbMV) complexes with ss-vDNA and ds-vDNA presumably in the nuclei
101 of infected cells, whereas NSP from *Squash leaf curl virus* (SLCV) binds only ss-vDNA *in vitro*
102 [9, 10, 11]. NSP also interacts with MP in the cytoplasm [12], which promotes the directionality
103 of virus translocation to the cell surface [12, 13]. The mechanism for vDNA exit from the nucleus
104 remains unsolved. It has been conceptually accepted that NSP facilitates the nuclear exit of newly-
105 replicated vDNA via nuclear pores [10, 14-17]. However, the interactions with the host nuclear
106 transport machinery have not been documented. A piece of this puzzle was obtained with the
107 identification of an NSP-interacting GTPase (NIG), which interacts with NSP at the cytosolic side
108 of the nuclear pore complex and facilitates the release of NSP-vDNA complex from the nuclear

109 envelope into the cytosol [12, 18]. At this step, it has not been resolved whether NSP-vDNA is
110 free in the cytosol or is transported via vesicles at their protoplasmic leaflet to the periphery.
111 Consistent with this transport function, the cytosolic NIG (i) accumulates around the nuclear
112 envelope, (ii) promotes the translocation of NSP-vDNA from the nucleus to the cytoplasm, (iii)
113 functions as a pro-viral factor during begomovirus infection and (iv) shares structural features and
114 transport properties with the human Rev-Interacting Protein (hRIP), which has been shown to be
115 involved in the release of HIV-1 RNAs from the nuclear periphery to the cytoplasm [12, 18, 19].
116 Histone H3 has been shown to bind NSP and MP from BDMV as an additional component of the
117 vDNA-protein complex that traffics intracellularly and intercellularly [20]. A nuclear
118 acetyltransferase (AtNSI) interacts with the *Cabbage leaf curl virus* (CabLCV) NSP and may
119 regulate the nuclear export of ss-vDNA-NSP via acetylation of CP and histones [21, 22]. Other
120 NSP-interacting partners include the receptor-like kinases (RLKs) NSP-interacting kinase (NIK1)
121 and NSP-associated kinase (NsAK), which are not involved in intracellular movement of vDNA
122 but rather are implicated in antiviral immunity against begomoviruses [23-26].

123 For the cell-to-cell transport of vDNA, two distinct mechanisms, which basically differ in
124 the nature of the vDNA-protein complex translocated into adjacent cells, have been suggested [11,
125 27]. In the first mechanism, designated "relay race" model, NSP facilitates the translocation of
126 viral DNA from the nucleus to the cytoplasm and then is replaced by MP, which promotes the cell-
127 to-cell movement of vDNA via plasmodesmata [10, 14]. The second mechanism represented by
128 the "couple-skating" model preconizes that MP facilitates the vDNA-NSP nucleocytoplasmic
129 trafficking and promotes the translocation of vDNA-NSP complex into the adjacent cells via
130 endoplasmic-reticulum-derived tubules induced by the viral infection [11-13, 18, 28-32]. More
131 recently, an alternative route for intracellular translocation and cell-to-cell spread of vDNA via

132 chloroplasts and stromules has been proposed, which was based on experimental data of AbMV
133 MP interactions (33,34). AbMV infection has been shown to induce the formation of a stromule
134 network interconnecting different chloroplasts, chloroplasts with nuclei, and expanding to the cell
135 periphery presumably at plasmodesmata [34]. This stromule network is stabilized by the plastid
136 chaperone cpHSC70, which has also been shown to interact with MP [33]. The interaction between
137 MP and cpHSC70 may bridge viral nucleoprotein complexes with stromules. Independent on the
138 cell-to-cell transport models, the underlying mechanisms of the intracytoplasmic retrograde and
139 anterograde movements of vDNA complexes remain to be determined. CabLCV and SLCV MPs
140 have been shown to interact with synaptotagmin A (SYTA), which is located in the endosomes of
141 plant cells and is required for the cell-to-cell trafficking of different MPs [35]. These findings
142 raised the hypothesis that distinct viral MPs promote an intracytoplasmic transport of vDNA-
143 complexes to plasmodesmata via an endocytic recycling pathway. Likewise, the AbMV MP has
144 been shown to interfere with microtubule assembly and stability when co-expressed in plant tissues
145 with a viral inducer of cell progression, which may suggest a microtubule-associated transit of MP
146 or begomoviruses to the plasmodesmata [36]. However, the host components of the vDNA
147 complex competent for intracytoplasmic translocation have not been identified, and the underlying
148 mechanism for intracytoplasmic trafficking of vDNA remains to be elucidated.

149 In this investigation, we used a protein microarray assay to identify MP- and NSP-host
150 protein-protein interactions (PPIs). From the NSP-MP-host PPI network map, we selected an
151 intracellular transport-associated protein, designated NSP-interacting syntaxin domain-containing
152 protein (NISP), as an NSP specific target, to gain further insight into the intracytoplasmic transport
153 of begomoviruses. We showed that NISP, which was located in motile vesicle-like structures,
154 many of which were targeted to the endosomes, interacted with NSP *in vivo* and displayed a pro-

155 viral function. Furthermore, NISP also interacted with NIG, and the complex formed was enhanced
156 by the presence of viral NSP. In addition, vDNA might have been recruited into the NISP complex.
157 Our data indicate that NISP might be part of the vDNA complex that traffics intracellularly via
158 endosomes.
159
160
161 **Results**
162
163 **Identification of Arabidopsis proteins interacting with *in vitro* transcribed and translated**
164 **MP and NSP**
165 To search for the begomovirus movement proteins-Arabidopsis protein-protein
166 interactions (PPIs), we used a nucleic acid programmable protein array (NAPPA)-based approach
167 [37], in which we interrogated 4600 *in vitro* synthesized ORFs from Arabidopsis for their capacity
168 to be targeted by MP and/or NSP from CabLCV. The *in situ* synthesized NAPPA protein
169 microarray has previously generated a transcription factor (TF)-NAPPA dataset, which has been
170 validated by pull-down assays and bimolecular fluorescence complementation (BiFC) assays in *N.*
171 *benthamiana* leaves using a subset of newly identified interactions [37]. NSP and MP were *in vitro*
172 transcribed and translated to probe this protein microarray (S1a and S1b Fig). A total of 40
173 candidate proteins were identified each for MP and NSP. The majority (35 proteins of 40) were
174 found to interact with both proteins, which was not surprising because MP itself interacts with
175 NSP and may be part of common multiprotein complexes as both participate in viral DNA
176 movement (S2 Fig). We found five new NSP-specific protein interactions (dark orange) and five
177 MP-specific protein interactions (light yellow), including a peptidyl-prolyl cis-trans isomerase

178 family protein (AT1G73655) belonging to the same protein family as Pin4, which has been shown
179 previously to interact with MP (S1 Table) [36]. As previously identified NSP-interacting proteins
180 were absent in the protein array, the identification of known MP-specific interacting protein
181 contributed to validate our current data.

182 The protein-protein interactions between the viral proteins and the Arabidopsis proteins
183 were integrated into the protein-protein interactome experimentally determined for *Arabidopsis*
184 *thaliana* (BioGRID database and the Arabidopsis interactome database), using Cytoscape
185 software. This procedure identified the PPI network containing MP, NSP and all, directly and
186 indirectly, interacting host proteins (Fig 1). AT1G68185, an ubiquitin-like protein, which formed
187 a large hub (hub 20, degree 148), may represent a convergent targeting node among begomovirus
188 viral proteins as it was found to be a direct target of NSP and MP independently (S1 Table).
189 Furthermore, the ubiquitin-proteasome pathway (UPS) is targeted by many viruses to maintain
190 suitable levels of viral proteins and to induce, inhibit or modify ubiquitin (Ub)-related host proteins
191 [38]. Other MP-interacting proteins, including FKBP-like peptidyl-prolyl cis-trans isomerase
192 family protein (this work), chloroplast heat shock protein 70-1 [33] and synaptotagmin A [35],
193 which have been shown to assist the MP movement function, are connected to the AT1G68185
194 hub (hub 20), further supporting the notion that this hub may be biologically significant during
195 begomovirus infection.

196 To gain further insights into the cellular processes affected by NSP or MP, we performed
197 functional enrichment analyses of their direct and indirect interactors. Significantly enriched GO
198 terms (with p-value < 0.05) were identified in all three categories, Biological Process, Molecular
199 Function, or Cellular Component ontology (S2 Table). Some over-represented GO terms in this
200 set of viral protein-interacting host proteins are consistent with NSP and MP function or

201 localization. Consistent with their function as viral movement proteins, a subset of host interactors
202 was significantly over-represented under the transport activity term and protein binding term in
203 Molecular Function (S2 Table). Under the cellular component ontology, we observed an over-
204 representation of proteins under membrane-bounded organelle term, intracellular vesicle term, and
205 SNARE complex term, which may suggest an intracytoplasmic route for begomovirus proteins
206 and vDNA trafficking (S2 Table). Accordingly, two NSP-interacting proteins, AT5G23550 and
207 AT4G30240, form convergent hubs (17 and 19) enriched for membrane-bound proteins and
208 transport activity function (Fig 1, S1 Table). The NSP-interacting protein AT5G23550 (hub 17)
209 is annotated as a Got1/Sft2-like vesicle transport protein, which may be located to a late-Golgi
210 compartment and may be involved in the fusion of retrograde transport vesicles derived from an
211 endocytic compartment with the Golgi complex [39]. Likewise, AT4G30240 (hub 19) is a soluble
212 N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-like protein harboring a
213 syntaxin-6 domain typical of proteins found in the endosomal transport vesicles [40].

214 Under the biological process ontology, the response to auxin term represented a
215 significantly enriched GO term, which has not been previously associated with begomovirus
216 infection (S2 Table). The NSP-MP-host PPI network uncovered two relevant hubs enriched for
217 auxin response-related proteins, which are represented by an NSP-MP general interaction-derived
218 hub (Fig 1, hub 9) and an NSP-specific host interaction-derived hub (hub 11). Remarkably, these
219 two viral protein hubs are interconnected to each other via interactions with proteins (in gray),
220 which are also over-represented under the auxin response term, forming a large auxin affected hub
221 target by begomovirus (S1 Table). This hub may represent an undocumented biological process
222 affected by begomovirus infection. The resulting PPI network map may provide a framework for

223 future studies in detailing the begomovirus complex life cycle. One such example is described
224 here, as following.
225
226 **A SNARE-like protein designated NSP-Interacting Syntaxin domain-containing Protein**
227 **(NISP), but not its paralog, interacts with NSP via the syntaxin domain**
228 The NSP-MP-Host PPI network uncovered enriched GO terms linked to intracellular
229 vesicle and membrane-bound proteins, which may be associated with a mechanism for
230 intracytoplasmic translocation of viral proteins and vDNA. To pursue further with this possible
231 connection and to validate further the microarray data, we selected a new specific NSP-interacting
232 protein, the SNARE-like protein AT4G30240, which harbors a syntaxin-6 domain at the N-
233 terminus and a transmembrane segment at the C-terminus, for further analyses (Fig 2a). SNARE-
234 complexes drive the fusion of membrane-bound vesicles with target membranes required for
235 intracellular transport via the endosome system. We used the syntaxin domain superfamily as the
236 prototype for the identification of homologs in the genomes of *Arabidopsis thaliana*, *Oryza sativa*,
237 *Drosophila melanogaster*, *Homo sapiens,* and *Saccharomyces cerevisiae*. We then selected the
238 most related proteins to construct phylogenetic trees using Bayesian inference method (S3a Fig).
239 AT4G30240 was clustered in pair with AT2G18860 and formed a clade of syntaxin-6 domain-
240 containing proteins, which also included a third Arabidopsis paralog AT1G27700. This
241 AT4G30240-derived clade (in red), also designated Syntaxin-6-like subgroup, clustered only plant
242 proteins, carrying an N-terminal syntaxin-6-like domain and a C-terminal transmembrane
243 segment. AT4G30240 and AT2G18860 may be a result of genome duplication, as these syntaxin-
244 6-like proteins clustered in pair even in phylogenetic analyses that include proteins from other

245 organisms, share a high degree of sequence conservation and display similar intron/genomic DNA
246 configuration (https://www.arabidopsis.org/).

247 The remaining syntaxin-6-like proteins clustered together in a distinct clade that included
248 proteins from human, rice and the Arabidopsis AT1G28490, also named SYP61, a characterized
249 trans-Golgi network (TGN)/endosome localized syntaxin [41-43]. This SYP61 second clade (in
250 orange) was designated target (t)-syntaxin-6/SNARE-like subgroup, as it included structurally
251 similar proteins carrying the N-terminal syntaxin-6 domain and also a t-SNARE coiled-coil
252 homology domain at the C-terminus.

253 However, the syntaxin-6-like subgroup represents a newplant-specific class in the t-
254 SNARE superfamily, since it lacks the coiled-coil t-SNARE domain at the C-terminus, which is
255 responsible for vesicle fusions. A function of this subgroup has to be determined, yet. This
256 hypothesis was further confirmed in a phylogenetic analysis of syntaxin-6 domain-containing
257 proteins from *Arabidopsis thaliana, Oriza sativa*, *Glycine max*, *Zea mays*, *Zostera marina*, *Homo*
258 *sapiens*, *Mus musculus*, *Bos taurus*, *Equus caballus*, *Danio rerio*, and *Drosophila melanogaster*
259 using the full-length sequence of AT4G30240 as prototype (S3b Fig). The syntaxin-6 domain-
260 containing proteins form two distinct clades with high internal stability (100%). The AT4G30240-
261 derived clade, syntaxin-6-like clade, includes only plant syntaxin-6-like proteins.

262 To complement the protein microarray data, we used the yeast two-hybrid system to
263 examine further the NSP-AT4G30240 interaction and compared the paralog AT2G18860 in our
264 assays, which was not present in the protein microarray. The second most closely related paralog,
265 AT1G28490, was present in the protein microarray but was not found to interact with NSP. Co-
266 expression of the full-length AT4G30240 (NISP) ORF fused to the activation domain (AD), and
267 NSP fused to the binding domain (BD) of GAL4 promoted histidine prototrophy (Fig 2b). The

268 HIS3 marker gene was not activated in yeast cells cotransformed with the controls. In contrast,
269 AT2G18860 failed to interact with NSP in yeast, as neither AD-AT2G18860 nor BD-AT2G18860
270 promoted his prototrophy when cotransformed with the respective cognate BD-NSP or AD-NSP
271 (Fig 2c).

272 To map the NSP-interacting domain on NISP, we generated NISP deletion fragments and
273 assay for protein interactions by two-hybrid assay. The N-terminal NISP fragment extending up
274 to amino acid position 107 and containing an intact syntaxin-6 domain ($NISP^{1-107}$, Fig 2a) was
275 sufficient to bind NSP in yeast (Fig 2d). The interaction of the truncated $NISP^{1-107}$ with NSP was
276 specific because the HIS marker gene was not activated in yeast cells co-transformed with AD- or
277 BD-truncated $NISP^{1-107}$ and the empty vectors (Fig 2d). In contrast, the NISP truncated versions,
278 comprising the NISP internal region, delimited by positions 105-200 ($NISP^{105-200}$; Fig 2e) or the
279 C-terminal portion ($NISP^{200-300}$; Fig 2f), were not capable of recognizing NSP, confirming that
280 NISP binds NSP via its syntaxin-6 domain. Although the syntaxin-6 domains of NISP and
281 AT2G18860 are conserved (78.57% identity), NSP interacts specifically with NISP in yeast (Fig
282 2b). Sequence alignment revealed an 8-amino acid deletion at the syntaxin-6 domain of
283 AT2G18860 as the most striking feature that may account for the differences in protein binding
284 specificity (S4 Fig).

285 Although NISP lacks a typical coiled-coil t-SNARE domain, it harbors a syntaxin-6
286 domain at the N-terminus and a predicted transmembrane segment at the C-terminus, which
287 suggests an endomembrane-bound localization (Fig 2a). Mammalian syntaxin-6 domain proteins
288 are localized in the trans-Golgi network (TGN) membranes and endosomal vesicles [44]. To
289 examine the subcellular localization of NISP, we transiently expressed AT4G30240 fused to a
290 green fluorescent protein (GFP) under the control of the native and constitutive promoters, in

291 *Nicotiana benthamiana* leaves and analyzed the localization of the fluorescent signal by confocal
292 microscopy. NISP-GFP was predominantly localized in motile spherical structure, resembling
293 vesicles (Fig 3a, 3b; S1 Video). The paralog AT2G18860 fused to GFP appeared concentrated
294 predominantly in membrane-bound vesicles and plasma membrane (S5 Fig).

295 To examine more precisely the NISP localization, we performed co-localization assays,
296 using SYTA-mCherry as an endosome-associated marker [35], the plasma membrane-associated
297 molecular marker FLS2-mCherry [45], ER-, TGN- and tonoplast-associated molecular markers
298 constructed by Nelson et al. [46]. NISP-GFP did not co-localize with ER-mCherry- (Fig 3c,
299 asterisks indicate the field of the insets) and tonoplast (vac-mCherry)- (Fig 3e) associated
300 molecular markers. Likewise, NISP-containing vesicles did not co-localize with the plasma
301 membrane FLS2-mCherry (Fig 3f). In contrast, a small population of NISP-GFP-containing
302 vesicles co-localized with the TGN-associated molecular marker (Fig 3d) and a large fraction of
303 NISP-GFP-containing vesicles precisely co-localized with the endosomal protein SYTA-mCherry
304 (Fig 3g). Because many of the NISP-labeled vesicles were associated with the plasma membrane,
305 we examined whether the NISP-associated vesicle membrane was derived from the plasma
306 membrane by staining NISP-GFP-expressing cells with FM4-64, a marker for plasma membrane
307 internalization that labels endosomes [47]. In addition to dye-labeled plasma membranes, some
308 vesicles showed also NISP-GFP fluorescence (Fig 3H, arrows). Collectively, these results indicate
309 that NISP localizes in TGN/early endosome vesicles.

310

311 **NISP interacts with NSP in intracellular vesicles**

312 We next examined whether NISP interacts with NSP *in vivo* using the bimolecular
313 fluorescence complementation (BiFC) assay. The formation of NISP/NSP complexes occurred

314 atvesicles of *N. benthamiana* epidermal cells independent of the orientation of the NISP fusions
315 (N-terminus or C-terminus of YFP; Fig 4a), and the reconstituted fluorescent signal was much
316 higher than that of the background (control panels with combinations of the protein fusions with
317 empty vectors). The reconstituted fluorescent signal was co-labeled by FM4-64, indicating that the
318 complex was formed in endosomal vesicles derived from the plasma membrane (Fig 4b, see
319 arrows). We further demonstrated that NISP and NSP interacted *in vivo* using co-
320 immunoprecipitation assays. Both NISP-GFP and NSP-6HA, were co-immunoprecipitated from
321 co-infiltrated extracts using anti-GFP antibody, demonstrating an association of NISP-GFP with
322 NSP-6HA fusions (Fig 4c). Switching the protein tags and including MP did not alter the results
323 because NISP-6HA also associated with NSP-GFP in co-infiltrated extracts (Fig 4d). Co-
324 expression of MP in the assay was used to mimic infection, since CabLCV MP promotes the
325 directionality of NSP to the cell periphery (12, 13). The Co-IP results also demonstrated that GFP
326 alone did not interact with either NSP-6HA or NISP-6HA, demonstrating the specificity of the
327 interactions between the viral protein and NISP.
328
329 **NISP, but not its paralog AT2G18860, displays a pro-viral function**
330 The biological relevance of the NSP-NISP interactions in begomovirus infection was
331 examined using several different approaches. We first analyzed the expression of NISP from
332 publically available RNA-seq data, which showed that NISP was induced by begomovirus
333 infection [26]. Quantitative RT-PCRs showed that NISP RNAs, but not the RNA from the NISP
334 paralog AT2G18860, were induced by the begomovirus infection (S6 Fig). We then identified a
335 T-DNA insertion mutant in the NISP gene (*nisp-1*) and the AT2G18860 gene (*at2g18860-1*). The
336 *NISP* or AT2G18860 transcript levels in the respective T-DNA insertion mutant lines were much

337 lower than in Col-0, indicating that *nisp-1* and *at2g18860-1* may be loss-of-function mutant lines
338 (Fig 5a, 5c). These mutant lines were transformed with *NISP-GFP* or AT2G18860-GFP constructs
339 to analyze a complementation of the defects in three independent lines each (Fig 5a, 5b, *nisp-*
340 *1*/NISP lines, and *at2g18860-1*/AT2G18860 lines). We also prepared transgenic lines
341 overexpressing *NISP-GFP* and AT2G18860-GFP, under the control of the 35S constitutive
342 promoter. The respective transcript (Fig 5a, 5c) levels were higher in NISP and AT2G18860 plants
343 than in Col-0 and accumulation of the encoding proteins was determined by immunoblotting with
344 an anti-GFP antibody (Fig 5b, 5d). The knockout and overexpressing lines were phenotypically
345 indistinguishable from Col-0 (S8 Fig).

346 We next examined whether *NISP* and AT2G18860 participate in begomovirus infection.
347 The knockout lines (*nisp-1* and *at2g18860-1),* complemented lines (*nisp-1/NISP* and *at2g18860-*
348 *1/AT2G18860), NISP*-overexpressing lines and Col-0 were inoculated with infectious clones of
349 CabLCV and the course of infection was monitored by visualization of symptom development and
350 PCR diagnosis. The accumulation of viral DNA was quantified by qPCR (Fig 5).

351 The *nisp-1* knockout line displayed attenuated symptoms (Fig 5e, S7 Fig), delayed course
352 of infection (Fig 5f), and lower viral DNA as compared to Col-0 (Fig 5g). Expression of NISP-
353 GFP in *nisp-1* restored the wild type susceptibility to begomovirus infection in all three
354 independently transformed lines (*nisp-1/NISP*), confirming that the reduced susceptibility
355 displayed by the *nisp-1* line was indeed due to loss of *NISP* function. In contrast, *NISP*
356 overexpression conferred enhanced susceptibility to begomovirus infection; the progress of
357 infection was accelerated (Fig 5f), and viral DNA load was significantly higher in the *NISP*-
358 overexpressing lines than in Col-0 and *nisp-1* lines (Fig 5g). We predicted that if NISP-NSP
359 binding was the basis for the NISP pro-viral function, the closest paralog of NISP, AT2G18860,

360 which harbors the syntaxin-6-like domain but does not interact with NSP, would not affect
361 begomovirus infection. Accordingly, the *at2g18860-1* knockout line, *at2g18860-1/AT2G18860*
362 complemented lines, and an *AT2G18860*-overexpressing line displayed similar symptoms, course
363 of infection and viral DNA load as Col-0, indicating that the presence or absence of the
364 *AT2G18860* gene does not change the begomovirus infection (Fig 5e, 5f, 5g, S7 Fig). These results
365 further substantiated a specific pro-viral function of NISP rather than of syntaxin-6 like domain
366 proteins in general.
367

368 **NISP also interacts with NIG and complex formation is enhanced by viral NSP**

369 There is a gap in our knowledge of how the vDNA-NSP complex, which binds to NIG in
370 the cytosolic side of the nuclear pores, traffics to the cell periphery. From the PPI network map,
371 we hypothesized that NIG could serve as a bridge to attach vDNA to the intracellular transport
372 system of plant cells. The integration of our data with the Arabidopsis interactome uncovered a
373 possible interaction between NIG and NISP, which was also shown here to bind specifically to
374 viral NSP. The interaction between NIG and NISP was first demonstrated by two-hybrid assay
375 (S9a Fig). The co-expression of the NISP ORF fused to BD and NIG ORF fused to AD from GAL4
376 promoted histidine prototrophy in medium supplemented with 2.5 mM 3AT, whereas co-
377 expression with empty vectors did not.

378 To examine whether NIG and NISP interact *in vivo*, we used BiFC and co-
379 immunoprecipitation assays. NIG has been shown to be unevenly distributed around the nuclear
380 envelope, and cell periphery (S9b and S9c Fig) [25, 48]. In the BiFC analyses, nYFP-NIG as well
381 as cYFP-NISP (and vice-versa) were localized at the cell periphery (Fig 6a), probably at the NISP
382 endosomes, since the structures were co-stained by FM4-64 (Fig 6b; arrows). The fluorescent

383 signal was barely detected in the controls (Fig 6a). The inclusion of NSP-6HA in the BiFC assay
384 increased the NISP-NIG complex formation, as judged by the enhanced intensity of the
385 reconstituted fluorescent signal, showing the fluorescent vesicles associated with the plasma
386 membrane (S10 Fig). To provide further evidence of the NSP-mediated enhancement of NIG-
387 NISP interaction, we used a quantitative co-immunoprecipitation assay (Fig 6, S9 Fig). We first
388 confirmed that NIG interacts with NISP *in vivo* (Fig 6c, S10d). The Co-IP results indicated that
389 NISP-GFP, but not GFP alone, interacted with NIG-HA. Likewise, NIG-GFP, but not GFP alone,
390 interacted with NISP-HA. NSP promoted a stronger association of NISP-GFP with NIG-6HA, as
391 judged by the amount of co-immunoprecipitated NIG by anti-GFP in the presence of co-expressed
392 GST-NSP (Fig 6d, 6e). Likewise, GST-NSP enhanced NISP-6HA-NIG-GFP complex formation
393 (S9e and S9f Fig). These results indicate that NSP/NIG and NISP might form a multiprotein
394 complex at endosomes. Syntaxin-6 domain-containing proteins have been shown to participate in
395 the endosome-plasma membrane route of protein cargos.
396
397 **NISP complexes viral DNA in vivo**
398 The current model of vDNA intracellular translocation informs that NSP binds to nascent
399 viral DNA in the nucleus, facilitates its translocation to the cytosol via nuclear pores and moves
400 vDNA intracytoplasmically to the cell periphery, where MP mediates the intercellular movement
401 of vDNA-NSP or vDNA-MP via plasmodesmata. Because NSP also binds to NISP in vesicle-like
402 structures, we asked whether the vDNA would be recruited into a NISP multi-protein complex
403 using a modified chromatin immunoprecipitation (ChIP) assay (Fig 7). In our assay, proteins and
404 DNA were first cross-linked with formaldehyde and then the protein-DNA complexes were
405 immunoprecipitated with antibody (anti-GFP for NISP-GFP) from total protein extracts. The

406 untransformed line (Col-0) and NISP-GFP-overexpressing (AtNISP-1, AtNISP-2, and AtNISP-3,
407 Fig 7a) lines were infected with CabLCV infectious clone and the modified ChIP assay was
408 performed using an anti-GFP antibody. We confirmed first that the inoculated plants were infected
409 (IN) by performing PCR from input DNA using CabLCV-specific primers, which amplify a 770-
410 bp fragment from CabLCV DNA-B (Fig 7b and S11b Fig, primers F1/R1) and a 389-bp fragment
411 from DNA-A (Fig 7e and S11a Fig, primers F1/R1). This viral DNA-B and DNA-A fragments
412 were also amplified from ChIP-DNA of infected (IN) AtNISP-overexpressing lines but not of
413 infected (IN) or uninfected (UN) Col-0, which lacks GFP-tagged NISP (Fig 7a, 7e). Using two
414 sets of DNA-B-specific primers (F2/R2 and F3/R3, S10b Fig), ChIP-qPCR further demonstrated
415 significant enrichment of DNA-B-specific fragments in infected AtNISP-GFP-overexpressing
416 samples, which had been precipitated with anti-GFP serum, but not in pulled down samples from
417 infected Col-0 lines (Fig 7c and 7d). Likewise, the use of F2/R2 set of DNA-A primers (S10a Fig
418 10a) also resulted in significant enrichment of the DNA-A-specific fragment in ChIPed DNA from
419 infected AtNISP-GFP-overexpressing samples but not from infected Col-0 (Fig 7f). Collectively,
420 our results indicate that NISP is a functional component of the vDNA-complex that traffics along
421 the cytoplasm to the cell periphery for the MP-mediated cell-to-cell movement of vDNA into
422 adjacent cells.
423

424 **Discussion**

425

426 Viruses are obligate intracellular parasites and, once in the host cell cytoplasm, they must
427 move intracellularly to and from the replication site to the plasma membrane for spreading. The
428 cytoplasmic limitations on free diffusion have forced viruses to evolve efficient mechanisms to

429 manipulate the host transport system for their benefit. Understanding how viruses hijack the host
430 intracellular transport system using a limited repertoire of proteins provides an opportunity to
431 uncover the molecular bases of the host transport machinery. Bipartite begomoviruses, plant
432 ssDNA viruses, use two movement proteins, MP and NSP, to move intracellularly from the site of
433 replication in the nucleus to the cell surface [17]. However, the host components of the vDNA
434 complex competent for intracytoplasmic translocation have not been identified, and the underlying
435 mechanism for intracytoplasmic trafficking of vDNA remains to be elucidated. Here, we used a
436 previously fabricated, *in situ* synthesized protein microarray containing 4600 Arabidopsis ORFs
437 [37] to identify NSP- and MP-Arabidopsis protein-protein interactions (PPIs). Consistent with the
438 movement function of the viral proteins, the identified NSP-MP-PPI network uncovered direct and
439 indirect interactions, over-represented under transport activity, protein binding, membrane-bound
440 organelles, intracellular vesicle, and SNARE complex ontology terms, which may define an
441 intracellular route for vDNA trafficking. Precedents in the literature have implicated a
442 microtubule-associated intracellular movement of viral proteins and vDNA in addition to an
443 endocytic recycling pathway for the MP-mediated cell-to-cell movement of vDNA protein
444 complexes (35; 36]. Accordingly, transport function-related proteins, including kinesin motor
445 protein, plant syntaxin 121, Rab GTPase, Ras, Got1/Sft2-like vesicle transport protein, target
446 SNARE coiled-coil domain protein, syntaxin-6 plant proteins, prenylated Rab acceptor, Heat
447 shock protein 70 (Hsp 70), nuclear pore anchor, synaptotagmin A, were over-represented in the
448 MP-NSP- PPI network. These studies thus provided a critical framework for future investigations
449 to elucidate the molecular mechanisms for intracytoplasmic transport of begomoviruses.

450 **The NSP-interacting syntaxin domain-containing protein (NISP) is identified as a new**
451 **functional cellular partner of begomovirus NSP**

452 In the present investigation, we characterized an NSP-specifically interacting syntaxin-6
453 domain-containing protein, designated NISP and provided several lines of evidence indicating that
454 NISP might be involved in the intracytoplasmic movement of NSP-vDNA. First, co-localization
455 studies using organelle-associated molecular markers, and the FM4-64 marker demonstrated that
456 NISP is associated with TGN/early endosomal vesicles. Second, we confirmed that NISP
457 interacted with NSP *in planta* and showed that the complex formation occurred in vesicle-like
458 structures, which resemble the NISP-associated TGN/endosomal vesicles, the typical subcellular
459 localization of syntaxin-6-like proteins from Arabidopsis and humans [49]. Third, we showed that
460 NISP exhibits a pro-viral function, and begomovirus infection required NISP-NSP interaction. The
461 mutant *nisp-1* was less susceptible nt to begomovirus as the knockout lines displayed attenuated
462 symptoms, a delayed course of infection, and accumulated much lower viral DNA as compared to
463 Col-0. In contrast, the overexpressing lines were more susceptible to begomovirus. We took
464 advantage of a highly conserved NISP paralog, AT2G18860, to show that NISP-NSP interaction
465 was the molecular basis for the NISP pro-viral function. AT2G18860, which shares with NISP a
466 78,6% identical syntaxin-6 domain, did not interact with NSP and thus did not interfere with
467 begomovirus infection. Consistent with these data, NISP, but not AT2G18860, is induced by
468 begomovirus infection. Fourth, NISP also interacted with NIG, which facilitates the release of the
469 NSP-DNA complex from the nuclear pores to the cytosol [12,18], and the presence of NSP
470 enhanced the NISP-NIG complex formation, indicating that NSP, which interacts with NIG [12]
471 and NISP (Fig 4), may be recruited by the complex. We used ChIP assay to demonstrate that NISP
472 was also associated with vDNA in infected cells, which may be assembled into a NISP-NIG-NSP
473 multiprotein complex. Finally, the NISP interactions relocated the dispersed cytosolic NIG and
474 viral NSP to trafficking vesicle resembling the NISP-containing spherical structure associated with

475 TGN/endosome. The NISP-mediated translocation of NSP-vDNA to endosomal vesicles favors
476 the association of the viral complex with MP, which is brought to the endosome via interaction
477 with the endosome-associated SYTA for the MP-mediated cell-to-cell movement of vDNA [35].
478

479 **NISP belongs to a new, previously unidentified plant-specific subfamily of syntaxin-6-like**
480 **proteins**

481 The phylogenetic reconstruction of syntaxin-6 domain-containing proteins from different
482 eukaryotic organisms divided this family into two subclades, one containing proteins from all
483 tested organisms, including SYP61 from Arabidopsis, and a new, previously unidentified plant-
484 specific subclade, which includes NISP and AT2G18860 paralogs clustered in pair. SYP61 has
485 been shown to form functional SNARE complexes involved in the vacuolar-TGN recycling
486 pathway and in exocytotic trafficking to the plasma membrane [43,50]. The process of membrane
487 fusion is the final step of vesicle transport, which is generally driven by a specific pairing of 1 v-
488 SNAREs with the 2 or 3 cognate t-SNARE molecules into a trans-SNARE complex [49, 51]. The
489 proteins from the SYP61 subclade contain the typical structural configuration of t-SNARES,
490 including the N-terminal syntaxin-6 domain, followed by a C-terminal t-SNARE coiled-coil
491 homology domain and a transmembrane segment at the C-terminus. The proteins from the NISP
492 subclade harbor the N-terminal syntaxin-6 domain and the transmembrane segment but lack a C-
493 terminal SNARE domain, which may impair the participation of these new syntaxin-6-like proteins
494 in the assembly of a functional trans-SNARE complex for membrane fusion. Nevertheless, we
495 have demonstrated that a representative of this new syntaxin-6 subclade, NISP, is functionally
496 expressed in plant cells. In addition to being induced by begomovirus infection, NISP functions as
497 a susceptibility factor to begomoviruses, forms complexes with viral and host proteins and is

498 located in TGN/endosome-associated vesicles. We also mapped the NSP-binding domain on NISP
499 to the syntaxin-6 domain. Therefore, we propose that NISP may function as an intracellular vesicle
500 docking site to which the cargo proteins are specifically recruited through interactions with the
501 syntaxin-6-like domain.

502 We also showed that members of the new syntaxin-6 subclade might diverge functionally
503 concerning protein-protein binding specificity. The NISP paralog, AT218860, which shares with
504 NISP a conserved syntaxin-6 domain (78.6% identity), fails to interact with viral NSP and hence
505 does not affect begomovirus infection. Therefore, specificity of protein interactions may rely on
506 marginal features of the syntaxin-6 domain. A striking difference between NISP and AT2G18860
507 is an eight-amino acid deletion toward the N-terminus of the syntaxin-6 domain, which may
508 account for the NISP specificity to NSP (S4 Fig). Complementary studies are necessary to
509 precisely define the determinants of a functional syntaxin-6 domain for NSP interaction, which
510 will be useful for engineering resistance to begomoviruses.

511

512 **NISP may participate in the intracytoplasmic transport of NSP-vDNA**

513 Based on the current data and others previously reported data, we propose a model for the
514 NISP integration into an intracytoplasmic transport route used for the begomovirus anterograde
515 movement. Begomoviruses replicate in the nuclei of infected cells, where NSP interacts with
516 newly-synthesized vDNA and facilitates the translocation of vDNA to the cytoplasm via nuclear
517 pores [13, 32]). This nuclear exportation process may be mediated by an exportin-like protein yet
518 to be identified. At the cytosolic side of the nuclear envelope, NIG associates with the NSP-vDNA
519 complex to accessorize the release of NSP-vDNA from the nuclear pores into the cytosol [12,18].
520 The pro-viral function of NIG is associated with its cytosolic localization because the WWP1-

521 mediated recruitment of NIG into nuclear bodies impairs begomovirus infection [48]. At the
522 cytosol, we showed here that NISP may recruit the NIG-NSP-vDNA complex to TGN/endosome-
523 associated transport vesicles. The nature of the NISP-associated vesicle was identified using cell
524 biology molecular markers and FM4-64, which demonstrated that NISP is predominantly localized
525 at TGN and in the early endosome (Fig 3), which was consistent with the localization of syntaxin-
526 6 proteins in most eukaryotic cells [40]. As MP has been demonstrated to associate with the
527 endosomal SYTA, a possible NISP sequestration of NSP-DNA into TGN/endosome-associated
528 vesicles may provide the means for the predicted interactions between endosomally localized MP-
529 SYTA and NSP-DNA, which would favor the MP-mediated intercellular transport of vDNA via
530 an endocytic recycling pathway [35]. In summary, our data support a model in which direct
531 interactions between the host factor NISP and NIG/NSP modulate vDNA intracellular trafficking
532 to endosomes where MP, recruited by the endosomal SYTA, opportunistically interacts with NSP-
533 vDNA to mediate the cell-to-cell movement of vDNA via plasmodesmata.
534

535 ## Material and Methods

536

537 **Phylogenetic analysis**

538 The amino acid full-length sequences of plant proteins were retrieved from TAIR
539 (http://arabidopsis.org/), Phytozome v12 database (https://phytozome.jgi.doe.gov/) and amino acid
540 full-length sequences of other species from the Ensemble database
541 (https://www.ensembl.org/index.html). The syntaxin domain superfamily was predicted using the
542 software HMMER v3.2.1 (http://hmmer.org/) using Pfam v.30 database (https://pfam.xfam.org/).
543 The syntaxin domain-containing protein sequences were selected to perform the phylogenetic

544 reconstruction. The amino acid sequences were aligned using the MUSCLE algorithm [52].
545 Phylogenetic trees were constructed using Bayesian Inference with MrBayes v3.2.2 [53] and mix
546 model of amino acid substitution (Wag). The analyses were performed running 10.000.000
547 generations and excluding the first 2.500.000 generations as burn-in and visualized with Figtree
548 software (http://tree.bio.ed.ac.uk/software/figtree/). Plant NISP homologous proteins were
549 identified using the BLAST algorithm implemented in the Ensemble and Phytozome databases.
550 The amino acid sequences of the NISP homologs were selected for phylogenetic reconstruction of
551 N-terminal Syntaxin-6 family using the same tools and criteria applied for the superfamily
552 syntaxin. However, the amino acid substitution model Jones was used for the N-terminal Syntaxin-
553 6 phylogenetic tree.
554
555 **PPI network analysis**
556 NSP- and MP-interacting proteins from Arabidopsis were used as a query term to identify
557 their respective interactions described in the BAR database (Bio-Analytic Resource for Plant
558 Biology, http://bar.utoronto.ca/interactions/). The Biogrid and IntAct databases were selected for
559 searching. The protein-protein interactions (PPI) were retrieved and imported into the Cytoscape
560 software (https://cytoscape.org/) to visualize the topology of the PPI network and to calculate the
561 network centrality metrics for each protein. The measured network centralities were betweenness,
562 closeness, eccentricity, and degree. Briefly, the betweenness centrality in the PPI network of the
563 graph G = (V,E) was calculated by observing the number of times a node v (protein) contributes
564 as a link along the shorter paths among all nodes. The betweenness centrality of this node can be
565 analyzed as:
566

$$\sum_{s,t \in V} \frac{\sigma(s,t|v)}{\sigma(s,t)}$$

567 where V is the total set of nodes, $\sigma(s,t)$ is the number of shorter paths of (s,t), and $\sigma(s,t|v)$ is the
568 number of paths crossing v [54]. The betweenness centrality can indicate the relevance of a protein
569 as functionally capable of keeping together interacting proteins. Closeness centrality (of a node v
570 is the sum of the shortest path distances from w to all other nodes and calculated as described by
571 Freeman [55]:

$$\frac{1}{\sum_{w \in V} dist(v,w)}$$

574

575 A high closeness value can be determined by the fact that all nodes are usually close to node v.
576 The eccentricity centrality of a node v is the maximum distance from v to all other nodes in graph
577 G and can be analyzed as described by Hage and Harary [56]:

578

579 $$\frac{1}{max\{dist(v,w) : w \in V\}}$$

580

581 In addition, the degree centrality of node v was calculated by $\sum i{=}1^{K} di$, where d represents each
582 adjacent node and K, the total number of adjacent nodes. Nodes with a high degree can be
583 designated hubs and hold together different nodes with a lower degree.

584 **GO analysis of the PPI network**

585 The gene set enrichment analysis (GSEA) of the proteins that compose the PPI network
586 was performed using the Bioconductor GOstats package [57]. GSEA analysis computes
587 hypergeometric test p values for over- or underrepresentation for each GO term, and the annotation
588 of genes is based on the Bioconductor annotation package org.At.tair.

589

590 **Plasmid constructions**

591 All recombinant plasmids were generated by the GATEWAY cloning system (Invitrogen,
592 http://www.invitrogen.com/). The primers used for gene amplification are listed in S3 Table. The
593 coding regions of *NISP* (AT4G30240) and AT2G18860, with or without a translational stop codon,
594 were amplified from leaf cDNA of *Arabidopsis thaliana* ecotype Columbia (Col-0) and inserted
595 into the entry vectors pDONR201 and pDONR207. The resulting clones were designated
596 At4g30240-St-pDONR201 (pUFV2134), At4g30240-Ns-pDONR201 (pUFV2135), At2g18860-
597 St-pDONR201 (pUFV3193), At2g18860-Ns-pDONR201 (pUFV3194), At4g30240-St-
598 pDONR207 (pUFV2236), At4g30240-Ns-pDONR207 (pUFV2237), At2g18860-St- pDONR207
599 (pUFV3195), At2g18860-Ns-pDONR207 (pUFV3196). Then, the DNA inserts were transferred
600 by recombination from the entry vector to different yeast and plant expression vectors.

601 For the yeast two-hybrid assay, the *NISP* and AT2G18860 coding regions were transferred
602 from the respective entry vectors (At4g30240-St-pDONR201 and At2g18860-St-pDONR201) to
603 the yeast expression vectors pDEST32 and pDEST22, generated the clones At4g30240-St-
604 pDEST32 (pUFV2141), At4g30240-St-pDEST22 (pUFV2142), At2g18860-St-pDEST32
605 (pUFV3197) and At2g18860-St-pDEST22 (pUFV3198), fused to binding domain (BD)
606 (pDEST32) or activation domain (AD) (pDEST22) of GAL4. The *NSP*- and *NIG*-derived clones
607 used in the yeast two-hybrid assay have been previously described and designated NSP-St-
608 pDEST32, NSP-Cl-pDEST22 (Fontes *et al*., 2004), and NIG-pDEST22 (pUFV2547; Calil *et al*.,
609 2018).

610 For the subcellular localization experiments, the *NISP* and AT2G18860 coding region were
611 fused to GFP and expressed in planta under the control of the CaMV 35S promoter in the vector

612 pK7FWG2. For the co-immunoprecipitation assay, in addition to the GFP fusion, *NISP* ORF was
613 also fused to hemagglutinin (HA) tag, using the binary destination vector pK7m34GWfor three
614 fragment recombination. The resulting DNA constructions were At4g30240-pK7FWG2
615 (pUFV2576), At2g18860-pK7FWG2 (pUFV3199) and 2x35S::At4g30240-Ns-6HA-pH7m34GW
616 (pUFV2586).

617 For the subcellular co-localization assays, the ER-associated molecular marker ER-
618 mCherry (CD-959), the TGN-associated molecular marker G-mCherry (CD-967) and the
619 tonoplast-associated molecular marker vac-mCherry (CD-975) were obtained from the
620 Arabidopsis Biological Resource Center. The plasma membrane-associated molecular marker,
621 FLS2-mCherry was obtained by amplifying the *FLS2* ORF from Arabidopsis cDNA with the
622 primers AtFLS2-Fwd and AtFLS2-NS-Rvs (S3 Table), inserting the amplified fragment by
623 recombination into pDON207 and transferring it by triple recombination to the destination vector
624 pH7m34GW along with FLS2-Ns-pDON207, pDONR-P4-P1R+2x35S, and pDONR-P2R-
625 P3+mCherry, resulting in the clone FLS2-mCherry (pUFV3034). Likewise, the SYTA ORF was
626 amplified from leaf cDNA with the primers AtSYTA-Fwd and AtSYTA-NS-Rvs (S3 Table),
627 cloned into the entry vector pDONR-207 and transferred via triple recombination to the destination
628 vector pH7m34GW along with SYTA-NspDON207, pDONR-P4-P1R+2x35S, and pDONR-P2R-
629 P3+mCherry, generating SYTA-mCherry (pUFV3296).

630 The *NISP* coding region was also fused to GFP and expressed in plants under the control
631 of its endogenous promoter. For this, about 2000 bp of 5' flanking sequence of At4g30240 was
632 PCR-amplified using the primers described in S3 Table and cloned into pDONR-P4-P1R, yielding
633 proAt4g30240-pDONR-P4-P1R (pUFV2436). Then, the 5' flanking sequence was transferred by
634 triple recombination to the destination vector pK7Fm34GW along with At4g30240-Ns-pDON201,

635 proAt4g30240-pDONR-P4-P1R, and eGFP-pDONR-P2R-P3, resulting in the clone
636 proAt4g30240::At4g30240-GFP (pUFV2849). The *NSP*- and *NIG*-derived clones used for co-
637 immunoprecipitation assays have been previously described and designated as 2x35S-NSP-Cl-
638 6HA-pH7m34GW (pUFV2424), NSP-Cl-pK7FWG2(pUFV2536), 2x35S-NIG-Ns-6HA-
639 pH7m34GW (pUFV1946) and NIG- pK7FWG2 (pUFV1177) [48].

640 The SPYNE-GW and SPYCE-GW vectors, which contain the N-terminal (nYFP) and C-
641 terminal (cYFP) of YFP, respectively, were used for the BiFC experiments. At4g30240-Ns-
642 pDONR207 was transferred to SPYNE-GW and SPYCE-GW, yielding At4g30240-Ns-SPYNE
643 (pUFV2238) and At4g30240-Ns-SPYCE (pUFV2239). The *NSP*- and *NIG*-derived clones used in
644 the BiFC assays have been previously described and designated as NSP-Cl-Ns-SPYNE
645 (pUFV1654), NSP-Cl-Ns-SPYCE (pUFV1655), NIG-Ns-SPYNE (pUFV1646), and NIG-Ns-
646 SPYCE (pUFV1647) [48].

647 NISP truncated versions were obtained from At4g30240-St-pDONR201 by PCR using
648 primers listed in S3 Table. The generated fragments were cloned into the entry vector pDONR201
649 and designated as At4g30240(1-107)-St-pDONR201 (pUFV2806), At4g30240(105-200)-St-
650 pDONR201 (pUFV2807) and At4g30240(200-300)-St-pDONR201 (pUFV2808). The respective
651 DNA inserts were then transferred to the yeast expression vectors, yielding At4g30240(1-107)-St-
652 pDEST32 (pUFV2822), At4g30240(1-107)-St-pDEST22 (pUFV2819), At4g30240(105-200)-St-
653 pDEST32 (pUFV2823), At4g30240(105-200)-St-pDEST22 (pUFV2820), At4g30240(200-200)-
654 St-pDEST32 (pUFV2824) and At4g30240(200-300)-St-pDEST22 (pUFV2821).

655

656 **Detecting MP- and NSP-host protein interactions on high-density halo tag-NAPPAs**

657 The identification of NSP and MP partners was carried out through a previously fabricated
658 high-density HaloTag nucleic acid programmable protein array (HaloTag-NAPPA), as described
659 by Yazaki *et al*. [37]. The protein microarray, which consisted of a set of *in situ* synthesized
660 proteins from 4600 *Arabidopsis thaliana* ORFs, has been designated AtNAPPA01. 3×HA-fused
661 CabLCV MP and NSP proteins were independently expressed using the TNT system (Promega)
662 according to the manufacturer's recommendations. The *in vitro* synthesized HA-fused MP, and
663 HA-fused NSP were used to probe individually AtNAPPA01. The identification of MP- and NSP-
664 interacting proteins with the NAPPA protein-protein interaction method was from a single
665 experiment using duplicated spots on one glass slide. Interactions were considered positive only
666 when both replicated spots displayed normalized signal intensity above background. Experiments,
667 data scanning, quantification, and data processing were carried out as described by Yazaki *et al*.
668 [37].
669
670 **Plant growth conditions and transformation**
671 *Arabidopsis thaliana*, ecotype Columbia (Col-0), was used as the wild type. Homozygous
672 seeds from the T-DNA insertional mutants *nisp-1* (Salk_117751) and *at2g18860-1* (Salk_099699)
673 were obtained from the Arabidopsis Biological Resource Center. Arabidopsis seeds were surface-
674 sterilized and cold treated at 4°C for 72 h in the dark and then exposed to white light. Seedlings
675 were grown at 22°C on plates containing Murashige-Skoog (MS) medium for 2 weeks and then
676 transferred to soil. Plants were grown in a growth chamber at 22 ºC under short-day conditions
677 (12h light/12h dark). Transgenic lines were obtained via *A. tumefaciens*-mediated transformation
678 using the floral dip method [58]. Transformed plants with the indicated DNA constructions were
679 selected in MS medium supplemented with the appropriate antibiotic (kanamycin, pK7FWG2).

680 Selected transformants were confirmed by PCR, transferred to soil, and grown in a growth
681 chamber at 22 ºC under long-day conditions (16h light/8h dark) to generate seeds. For segregation
682 analysis, seeds were germinated on MS medium containing 50 μgmL^{-1} kanamycin. Homozygous
683 T1 lines for the T-DNA loci were selected by determining the frequency of their kanamycin-
684 resistant T2 seeds after self-pollination. Accumulation of NISP-GFP and AT2g18860-GFP
685 transcript and protein levels were monitored in each generation by qRT-PCR using gene-specific
686 primers (S3 Table) and immunoblotting using anti-GFP antibody. From the independently
687 transformed lines (8-15 lines of each construct and genotype transformed), we selected three
688 independently transformed lines which appeared to have an integrated T-DNA locus on a single
689 chromosome, since 75% of their T1 segregating seedlings were resistant to kanamycin (S4 Table).
690 The selected lines are presented in S4 Table. The nomenclature *nisp*-1/NISP-1-3 indicates that the
691 genotype *nisp-1* was transformed with At4g30240-pK7FWG2, harboring the *NISP* ORF fused to
692 the N-terminus of GFP under the control of the 35S promoter to generate NISP-complementing
693 lines. The designations AtNISP-1, AtNISP-2 and AtNISP-3 indicate three independently
694 transformed lines, which were generated by transformation of Col-0 with At4g30240-pK7FWG2
695 to obtain NISP-GFP-overexpressing lines. The same nomenclature was applied for the
696 transformation of *at2g18860-1* mutant and Col-0 with At2g18860-pK7FWG2, containing the
697 At2g18860 ORF fused to the N-terminus of GFP under the control of 35S promoter (S4 Table).
698

699 **RNA isolation and qRT-PCR analysis**

700 Primers were designed using the Primer Express 3.0 (Life Technologies). Total RNA was
701 extracted from Arabidopsis leaves using TRIzol reagent (Thermo Fisher Scientific) and quantified
702 with a NanoDrop spectrophotometer. Total RNA (2 μg) was treated with RNase-free DNase I and

703 reverse transcribed using oligo (dT) (0,5 μM) and 1U of M-MLV (Thermo). Real-time RT-PCR
704 was carried out using SYBR Green PCR Master Mix (Life Technologies), DNA template, and
705 primers in 10 µL of reaction and an ABI7500 real-time PCR system (Life Technologies). The
706 expression of each gene was normalized to the expression of *Actin* and quantified using the $2^{-\Delta Ct}$
707 method. The primers used to detect specific transcripts with real-time RT-PCR are listed in S3
708 Table.
709

710 **Yeast two-hybrid assay**

711 Competent cells of *Saccharomyces cerevisiae*, strain AH109 (MATa, trp1-901, leu2-3,
712 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-
713 GAL2TATA-ADE2, URA3::MEL1UAS-MEL1 TATA-lacZ), were cotransformed with the yeast
714 expression vectors pDEST22 (Gal4 AD) and pDEST32 (Gal4 BD), carrying the coding region of
715 the tested proteins, along with 100 µg of salmon sperm carrier DNA (ssDNA), using the lithium
716 acetate/polyethylene glycol (PEG) method. For the selection of double transformants, the cells
717 were plated on medium deficient in leucine and tryptophan (SD, Synthetic Dropout, -Leu, -Trp)
718 and cultured for 4 days at 28ºC. Interactions were monitored by the ability of the reporter strain to
719 grow on media lacking leucine, tryptophan, and histidine but supplemented with 2.5 mM 3-AT (3-
720 Amino-1,2,4-triazole; Sigma) for 4 days at 28ºC.
721

722 **Co-immunoprecipitation assay**

723 The *in vivo* interactions between NISP and NSP or NIG were monitored by co-
724 immunoprecipitation using the µMACS™ Epitope Tag Protein Isolation (MACS/Miltenyil Biotec)
725 kit, according to manufacturer's recommendations. *Agrobacterium tumefaciens (strain GV3101)-*

726 mediated transient expression in *N. benthamiana* leaves was carried out as described (12). Total
727 protein extracts were obtained from infiltrated leaves expressing the recombinant proteins. At 48
728 h after agro-infiltration, 200 mg of leaves were homogenized with 1 ml of lysis buffer (50 mM
729 Tris-HCl pH 8.0, 1 mM PMSF, 2mM benzamidine, 1% (v/v) Nonidet P-40) and incubated for 2 h
730 with anti-green fluorescent protein (GFP) magnetic beads (MACS / Miltenyi Biotec) at 4 ºC under
731 rotation. After incubation, the extracts were applied to a MACS column for the indicated protein
732 tag, washed five times before elution with a pre-heated buffer at 95 ºC. The immunoprecipitated
733 proteins were separated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-
734 PAGE) 10% (p/v), immunoblotted with the monoclonal antibodies anti-human influenza
735 hemagglutinin (anti-HA) epitope tag, amino acids 98-106 (Miltenyi Biotec, 130-091-972;
736 1:10.000) or anti-GFP (Miltenyi Biotec, 130-091-833; 1:10.000), and detected using Signal West
737 Pico Chemiluminescent Substrate (Thermo Scientific) according to the recommendations of the
738 manufacturer.
739
740 **Bimolecular fluorescence complementation (BiFC) assay**
741 Different combinations of *A. tumefaciens* strain GV3101 expressing the indicated proteins
742 fused to N-YFP/C-YFP were co-infiltrated into the abaxial surface of *N. benthamiana* leaves at
743 OD_{600nm} of 1:1 proportion. Three days after infiltration, fluorescence was examined in epidermal
744 cells using a Zeiss inverted 510 META laser scanning microscope equipped with an argon laser
745 and a helium laser as excitation sources. YFP was excited at 514 nm using the argon laser, and
746 YFP emission was detected using a 560–615-nm filter. The images were processed using LSM
747 Image Browser 4 (ZEISS) software.
748

749 **Subcellular localization**

750 The subcellular localization of proteins was examined in epidermal cells of *N. benthamiana*
751 leaves transiently expressing GFP fusions. Briefly, *N. benthamiana* leaves were agro-infiltrated
752 with *A. tumefaciens* strain GV3101 carrying proAt4g30240-pDONR-P4-P1R (pUFV2436),
753 At4g30240-pK7FWG2 (pUFV2576) or At2g18860-pK7FWG2 (pUFV3199) (Method S1) and
754 fluorescence of the GFP fusions were visualized in epidermal cells 3 days after infiltration by
755 confocal microscopy using a Zeiss inverted 510 META laser scanning microscope equipped with
756 an argon laser and a helium laser as excitation sources. GFP was excited by the argon/helium-neon
757 laser system in 488 nm wavelength, and emission was collected in 500–530 nm band-pass filter.
758 Images were captured and processed with LSM Image Browser 4 (Carl-Zeiss) software.

759 For co-localization assays, NISP-GFP was co-expressed with ER-, Golgi-, tonoplast-,
760 plasma membrane- and endosome-associated molecular markers fused to mCherry (see in plasmid
761 constructions) and fluorescence was examined by confocal microscopy, using a Zeiss inverted
762 LSM510 META laser scanning microscope, on whose system, the default scanning mode is
763 sequential scanning (multitrack mode). For imaging GFP, the 488 nm excitation line and the 500
764 to 530 nm band pass filter were used. Excitation of mCherry was at 540 nm and emission of 608-
765 680 nm. For the FM4-64 staining, 48 h after agro-infiltration with the DNA construct expressing
766 NISP-GFP, the leaves were infiltrated with a solution of 50 μM FM4-64 (Molecular Probes,
767 Eugene, OR) and examined by confocal microscopy 1h post-infiltration.

768

769 **Infectivity assay**

770 The generation of transgenic lines overexpressing NISP (AtNISp) and At2g18860
771 (AT2G18860), knockout lines (*nisp-1* and *at2g18860-1*), NISP complementing lines are described

772 in Method S1. Transgenic lines and Columbia at the seven-leaf stage were inoculated with
773 plasmids containing partial tandem repeats of CabLCV DNA-A and DNA-B by biolistic delivery
774 using a microprojectile bombardment model PDS-1000/He accelerator at 900 psi. (24, 26, 59). The
775 inoculated plants were transferred to a growth chamber and monitored for symptoms development
776 and progress of infection [23, 60]. Total DNA was extracted from systemically infected leaves
777 [61], and viral DNA accumulation was detected by PCR using universal DNA-B specific primers
778 PBL1v2040 and PCRC1 [62] (S3 Table). In each experiment, 15 plants of each line were
779 inoculated with 2 μg of tandemly repeated DNA-A plus DNA-B per plant. The course of infection
780 was examined using data from three independent experiments.
781
782 **Quantification of viral DNA in infected plants**
783 The accumulation of viral DNA in infected plants was quantified by qPCR [26]. The
784 reactions were prepared in 10 μL using SYBR Green Master Mix (Life Technologies), 100 ng of
785 total DNA from systemically infected leaves, and CabLCV-DNA-B-specific primers (S3 Table),
786 and analyzed on a real-time PCR system. The genomic copies of CabLCV were normalized against
787 an internal control (18S rRNA) to account for template input variation between tubes. For viral
788 DNA quantification, standard curves were prepared using serial dilutions of DNA-B of CabLCV
789 (100 to 10^6 copies of viral genome per reaction).
790
791 **Formaldehyde fixation and immunoprecipitation of NISP-GFP:vDNA complexes**
792 A modified ChIP assay evaluated the in vivo interaction between NISP and viral DNA.
793 NISP-overexpressing transgenic lines (AtNIS) and Columbia (Col-0) at the six-leaf stage were
794 inoculated by biolistic delivery with CabLCV DNA-A and DNA-B infectious clones. Tissue

795 harvest, formaldehyde fixation, and chromatin breaks from 45 days-old infected plants were
796 conducted according to Alves et al. [63] and Yamaguchi et al. [64]. For cross-linking proteins to
797 DNA with formaldehyde, 600 mg of infected leaves were washed with 10 mL of PBS (10 mM
798 Na2HPO4, 1.8 mm KH2PO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and fixed in 10 mL PBS by
799 adding formaldehyde to 1% final concentration. The infiltration of formaldehyde into vegetable
800 cells was carried out under vacuum at -500 Pa for 1.5 min, then 1 min twice with 30 s intervals.
801 After washing first with 0.125 M glycine under vacuum for 1.5 min and then with PBS, the tissue
802 was air-dried. The fixed samples were homogenized in liquid N2 and 1 mL of extraction buffer
803 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% (v/v) Nonidet-P40). After incubation for 30 min on
804 ice, cell debris was removed by centrifugation at 12000 x g for 10 min. The supernatant (300 µL)
805 was diluted with 700 µL of the ChIP dilution buffer (16.7 mM Tris-HCl pH 8,0, 167 mM NaCl,
806 1.2 mM EDTA, 0.01% SDS) and DNA was fragmented by sonication at an 80 µm amplitude level,
807 six pulses for 30 min, 30 s on/off on the ice. Then, 700 µL of the sonicated suspension was mixed
808 with 200 µL of the ChIP dilution buffer, supplemented with 1.1% (v/v) Triton X-100 and
809 centrifuged at 13200 x g for 5 min at 4° C. The supernatant was incubated with 50 µL of protein-
810 A-garose beads for 2 h. After centrifugation at 900 x g for 3 min, 18 µL of the supernatant were
811 stored at -80° C, as input control. The remaining supernatant was incubated with 1 µl of polyclonal
812 anti-GFP serum (Life Technologies A11122; 3x diluted) for 4h at 4° C. Anti-GFP-NISP-
813 GFP:vDNA complexes were captured by incubation with 50 µL of potein-A-garose beads for 4 h
814 at 4°C, under gentle agitation. After centrifugation at 900 x g for 3min, the pellet was washed five
815 times with different washing buffers in the order: twice with the washing buffer A (0.1% (w/v)
816 SDS 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8,1, 150 mM NaCl), once with
817 the buffer B (0.1% (w/v) SDS 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8,1,

818 500 mM NaCl), followed by the buffer C (250 mM LiCl, 1% (v/v) IGEPAL-CA630, 1% (w/v)
819 sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8,1) and finally with 0,5 X TE (10 mM
820 Tris-HCl pH 8,0, 1 M EDTA). The DNA pellet was reverse cross-linked from proteins and purified
821 using the QIAprep® Spin Miniprep kit.
822
823 **ChIP-qPCR**
824 NSP primers (S3 Table) were initially used to amplify viral DNA-B from the
825 immunoprecipitated DNA and input DNA to ensure DNA-B-specific amplification in samples.
826 Likewise, DNA-A-specific primers were used to examine initial amplification of the DNA-A
827 component. Each reaction contained 1 µL of immunoprecipitated DNA or 20-fold diluted input
828 DNA, 0.2 mM CabLCV-DNA-B-specific primers (S3 Table), and 5 µL of SYBR Green PCR
829 Master Mix (Life Technologies) in 10 µL total volume. The thermal cycling conditions of the
830 reactions consisted of an initial step at 95°C for 10 min, followed by 40 cycles at 94°C for 15 s
831 and 58°C for 45 s. The cycle threshold (Ct) values from Col-0 and AtNISP were normalized with
832 the Ct values of the respective inputs, and the results were expressed as a percentage of the input.
833
834 **Author Contribution**
835 B.C.G.M performed most of the experiments; L.G.C.M. performed the co-localization
836 experiments and protein localization assays; M.D.-B., A.Y.K. and J.Y. performed protein
837 microarray assay; B.C.G.M. and J.P.B.M. performed two hybrid assays; J.C.F.S. performed *in*
838 *silico* and statistical analyses; B.C.G.M. and A.A.S. performed BiFC assays; E.P.B.F. and J.R.E.
839 conceived experiments; B.C.G.M. and E.P.B.F. designed all experiments, analyzed the data and
840 wrote the manuscript.

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Competing interests

The authors have declared that no competing interests exist.

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1007

1008 **Figure Legends**

1009

1010 **Fig 1. MP-NSP-Arabidopsis protein-protein interactions (PPI) network**
1011 **integrated into Arabidopsis interactome.**

1012 The network displays a firework topology, which was assembled by the Cytoscape software. The
1013 viral proteins, MP and NSP, are represented in red. The NSP-specifically interacting proteins are
1014 in dark orange and connecting proteins from Arabidopsis in light orange. The MP-specifically
1015 interacting proteins are indicated in light yellow to which interacting dark yellow proteins
1016 converge. Arabidopsis proteins that interact with both MP and NSP are shown in green, forming
1017 hubs of convergent blue proteins. Gray proteins represent connecting points between two
1018 functionally similar hubs.

1019

1020 **Fig 2. NISP, but not AT2G18860, interacts with NSP via the Syntaxin-6 domain.**

1021 **(A)** Schematic representation of NISP and AT2G18860 sequence alignment. The syntaxin-6
1022 domain is indicated in green and the transmembrane segment, in red. The identity of sequence and
1023 positions of amino acid residues are indicated. Sequence alignment was performed with
1024 CLUSTAL OMEGA. **(B)** NISP and NSP interact in yeast. NISP and NSP fused to the activation

1025 domain (AD), and binding domain (BD) of Gal4 and also in combination with empty vectors were
1026 expressed in yeast. The interactions between the tested proteins were monitored by histidine
1027 prototrophy in medium supplemented with 3-AT for 4 days at 28°C. **(C)** AT2G18860 failed to
1028 interact with NSP in yeast. AT2G18860 and NSP fused to both Gal4AD and BD were expressed
1029 in yeast, and possible interactions were assayed in all combinations. Co-expressed NSP-BD and
1030 NISP-AD were used as a positive control. **(D-F)**. Mapping the NSP-binding domain on NISP.
1031 Yeast cells expressing the indicated truncated version of NISP and NSP fusions were plated on
1032 selective medium lacking leucine, tryptophan, and histidine and supplemented with 2.5 mM 3-AT
1033 and grown for 4 days at 28°C. The truncated versions of NISP are delimited by the indicated
1034 position of the amino acid residues in the full-length protein. Protein interaction was only observed
1035 by co-expressing AD-NISP$^{1-107}$ and BD-NSP.
1036

1037 **Fig 3. Subcellular localization of NISP.**

1038 **(A)** The NISP-GFP distribution in intracellular vesicles. *N. benthamiana* leaves were infiltrated
1039 with *A. tumefaciens* carrying a DNA construct expressing NISP-GFP under the control of 35S
1040 promoter. Confocal images from three independent experiments were taken 36 hours post-
1041 infiltration. Approximately 200 cells were examined. Scale bars, 10 μm and 20 μm. **(B)** Confocal
1042 images of NISP-GFP transiently expressed under the control of the endogenous promoter. Scale
1043 bars, 10 μm and 20 μm. **(C)** Co-expression of the ER-associated ER-mCherry molecular marker
1044 and NISP-GFP in *N. benthamiana* leaves. *N. benthamiana* leaf cells expressing the indicated
1045 fusion proteins were examined by confocal microscopy 2 days after infiltration. The asterisks
1046 indicate the localization of the amplified insets, and the arrows indicate the merged pattern of GFP-
1047 associated vesicles in C, D, E, F, G, H. The confocal images are representative samples from three

1048 independent experiments. Scale bars, 10 μm. **(D)** Co-expression of the Golgi-associated G-
1049 mCherry molecular marker and NISP-GFP in *N. benthamiana* leaves 2 days after agro-infiltration.
1050 Scale bars, 20 μm. **(E)** Co-expression of the tonoplast-associated vac-mCherry molecular marker
1051 and NISP-GFP in *N. benthamiana* leaves 2 days after agro-infiltration. Scale bars, 20 μm. **(F)** Co-
1052 expression of the plasma membrane-associated FLS2-mCherry and NISP-GFP in *N. benthamiana*
1053 leaves 2 days after agro-infiltration. Scale bars, 20 μm. **(G)** Co-expression of endosome-associated
1054 SYTA-mCherry and NISP-GFP in *N. benthamiana* leaves 2 days after agro-infiltration. Scale bars,
1055 10 μm. **(H)** Co-labeling of vesicles with NISP-GFP and FM4-64 in epidermal cells of *N.*
1056 *benthamiana.* Images were taken after 2 days post infiltration of *Agrobacterium* carrying a NISP-
1057 GFP expression vector and 1 h after infiltration of FM4-64. Scale bars, 20 μm.
1058

1059 **Fig 4. NISP interacts with NSP *in vivo.***

1060 **(A)** NISP interacts with NSP in vesicle-like compartments, as judged by BiFC. *N. benthamiana*
1061 leaf cells expressing NISP and NSP fused to the C-terminus (cYFP) or N-terminus (nYFP) of YFP,
1062 or in combination with the empty vectors, were observed by confocal microscopy 3 days after
1063 infiltration. Scale bars, 10 μm. The experiment was repeated at least four times with identical
1064 results. **(B)** NISP-NSP complex-containing vesicles are stained by FM4-64. *N. benthamiana* leaves
1065 were agroinfiltrated with nYFP-NISP- and cYFP-NSP-expressing DNA constructs. Images were
1066 taken 2 days post agroinfiltration and 30 min after infiltration of FM4-64. Arrows indicate
1067 examples of vesicle-associated reconstituted fluorescent signals that were co-stained by FM4-64.
1068 Scale bars, 10 μm. The experiment was repeated three times with similar results. **(C)** NISP interacts
1069 with NSP *in planta*. Whole cell protein extracts from N. benthamiana leaves expressing transiently
1070 NISP-GFP and NSP-6HA were used for co-immunoprecipitation assays using an anti-GFP serum.

1071 Input and IP show the levels of the expressed proteins NSP-6HA, NISP-GFP, and GFP. Anti-HA
1072 antibody was used to detect NSP-6HA from the immunoprecipitated complex. GFP was used as a
1073 negative control. Molecular mass markers are given on the left in kilodaltons. The experiment was
1074 repeated twice. **(D)** *In vivo* interaction of NISP with NSP. Co-immunoprecipitation was performed
1075 as described in C, except that the tags of the transiently expressed proteins were switched to NSP-
1076 GFP and NISP-6HA. The experiment was repeated twice.
1077

1078 **Fig 5. NISP displays a pro-begomoviral function.**

1079 **(A)** Transcript accumulation of NISP in transgenic lines. NISP transcript levels in three
1080 independently complemented transgenic lines (*nisp-1*/NISP-1-3), three independently transformed
1081 *NISP*-overexpressing lines (AtNISP1-3), Col-0 and *nisp-1* were determined by qRT-PCR. Gene
1082 expression was calculated using the $2^{-\Delta Ct}$ method, and actin was used as an endogenous control.
1083 Error bars represent 95% confidence intervals based on replicated samples (n=3) from three
1084 independent experiments. **(B)** NISP-GFP protein accumulation in transgenic lines. Whole cell
1085 protein extracts from the complemented transgenic lines (*nisp-1*/NISP), *NISP*-overexpressing lines
1086 (AtNISP), Col-0 and *nisp-1* were separated by SDS-PAGE and immunoblotted using an anti-GFP
1087 serum. **(C)** AT2G18860 transcript accumulation in transgenic lines. The transcript levels of
1088 AT2G18860 in complemented transgenic lines (*at2g18860-1*/AT2G18860-1-3), AT2G18860-
1089 overexpressing lines (AT2G18860-1-3), Col-0 and *at2g18860-1* were quantified by qRT-PCR and
1090 normalized to the actin as an endogenous control. Error bars indicate 95% confidence intervals
1091 based on replicated samples (n=3) from three independent experiments. **(D)** AT2G18860-GFP
1092 protein accumulation in transgenic lines. Total protein extracts from complemented transgenic
1093 lines (*at2g18860-1*/AT2G18860), AT2G18860-overexpressing lines (AT2G18860), Col-0 and

1094 *at2g18860-1* were fractionated by SDS-PAGE and immunoblotted using anti-GFP antibody. **(E)**
1095 CabLCV infection-associated symptoms in Arabidopsis genotypes at 21 days post-inoculation
1096 (dpi). The figure shows representative samples of mock-inoculated and CabLCV -infected plants.
1097 The genotypes are indicated in the figure and are the same as specified in A-D. **(F)** The onset of
1098 infection is delayed in *nisp-1* knockout line. Ecotype Col-0, *nisp-1*, *at2g18860-1*, *nisp-1*/NISP
1099 complemented lines and AtNISP-overexpressing lines (as in a-d) were infected with CabLCV
1100 DNA, and the course of infection was monitored as the percent of systemically infected plants at
1101 different dpi. **(G)** Absolute quantification of CabLCV genomic units in infected lines at 21 dpi.
1102 The genotypes are the same as specified in a-d. Error bars indicate 95% confidence intervals based
1103 on replicated samples from three independent experiments. Different letters indicate significative
1104 differences among the groups (Duncan test, $p < 0{,}05$, $n = 3$).
1105

1106 **Fig 6. NISP interacts with NIG *in vivo* and NSP enhances NISP-NIG complex**
1107 **formation.**

1108 **(A)** BIFC assay showing the interaction between NISP and NIG in vesicles of *N. benthamiana* leaf
1109 cells. *N. benthamiana* leaves transiently expressing NISP and NIG fused to the C-terminus (cYFP)
1110 or N-terminus (nYFP) of YFP were examined by confocal microscopy 3 days after infiltration.
1111 Scale bars, 10 µm and 20 µm. The experiment was repeated three times with similar results. **(B)**
1112 NISP-NIG complex-containing vesicles are stained by FM4-64. *N. benthamiana* leaves were
1113 agroinfiltrated with nYFP-NIG- and cYFP-NISP-expressing DNA constructs. Images were taken
1114 2 days post infiltration and 1 h after infiltration of FM4-64. Arrows indicate examples of vesicle-
1115 associated reconstituted fluorescent signals that were co-stained by FM4-64 and the asterisk, the
1116 region of the amplified inset. Scale bars, 10 µm. The experiment was repeated three times with

1117 identical results. **(C)** In vivo interaction of NISP and NIG. Total protein extracts from *N.*
1118 *benthamiana* expressing NISP-GFP and NIG-6HA were used for co-immunoprecipitation assays
1119 using anti-GFP. Input and IP show the levels of the expressed proteins NISP-GFP and NIG-6HA.
1120 Anti-HA was used to detect NIG-6HA from the immunoprecipitated complex. GFP was used as
1121 an unrelated protein. The experiment was repeated twice with identical results. **(D)** NISP-NIG
1122 complex formation in the presence of viral NSP. The Co-IP assay was performed as described in
1123 A, except that co-expressed NSP-GST was included in the assay. NSP-GST was detected by
1124 immunoprecipitating it from infiltrated leaves and immunoblotting with anti-GST. The experiment
1125 was repeated twice with identical results. **(E)** Viral NSP enhances the interaction between NISP
1126 and NIG. NIG levels in the immunoprecipitated complex in the presence and absence of viral NSP
1127 were quantified using the Band Analysis tools of the ImageLab software (Bio-Rad). The signal
1128 values were normalized using the IP NISP-GFP band, revealed in the same blot as the Co-IP band.
1129 A.U. denotes arbitrary units.
1130

1131 **Fig 7. NISP complexes with vDNA *in vivo.***

1132 **(A)** NISP-GFP accumulation in overexpressing lines. Whole cell protein extracts from the NISP-
1133 GFP-overexpressing lines, as indicated in the figure, and Col-0 were separated by SDS-PAGE and
1134 immunoblotted using an anti-GFP serum. **(B)** NISP binds to viral DNA-B *in vivo*. NISP-GFP-
1135 overexpressing lines (AtNISP-1, AtNISP-2, and AtNISP-3), and Col-0 were inoculated with
1136 infectious CabLCV clones prior to the ChIP assay, which was performed with leaf samples of the
1137 indicated genotypes using anti-GFP. The infected plants were confirmed by PCR of input DNA
1138 from inoculated plants using CabLCV DNA-B-specific primers, which amplifies a 770-bp
1139 fragment (F1/R1; S10b Fig). IN denotes infected leaves and UN, uninfected leaves. **(C)** ChIP-

1140 qPCR assay of leaves from AtNISP-GFP transgenic lines and Col-0. The samples were
1141 immunoprecipitated with an anti-GFP antibody and ChIPed DNA was quantified by qPCR using
1142 the DNA-B-specific primers F3/R3 (S10b Fig). Data were normalized relative to the input of each
1143 sample and are expressed as the percentage of input. (D) ChIP-qPCR assay using a different set of
1144 DNA-B-specific primers (F2/R2) and samples from the indicated genotypes. The ChIP-qPCR was
1145 performed as described in C. **(E)** NISP binds to viral DNA-A *in vivo*. NISP-GFP-overexpressing
1146 lines (AtNISP-1, AtNISP-2 and AtNISP-3), and Col-0 were inoculated with infectious CabLCV
1147 clones prior to the ChIP assay, which was performed with leaf samples of the indicated genotypes
1148 using anti-GFP. The infected plants were confirmed by PCR of input DNA from inoculated plants
1149 using CabLCV DNA-A-specific primers, which amplifies a 389-bp fragment (F1/R1; S10a Fig).
1150 IN denotes infected leaves and UN, uninfected leaves. **(F)** ChIP-qPCR assay using a set of DNA-
1151 A-specific primers (F2/R2, S10a Fig) and samples from the indicated genotypes. The ChIP-qPCR
1152 was performed as described in C. The above experiments were repeated three times with similar
1153 results.
1154

1155 **Supporting information**

1156 **S1 Table-** Protein-protein interactions between the viral proteins and the
1157 Arabidopsis proteins**.**
1158

1159 **S2 Table-** Enriched GO terms in three categories, Biological Process, Molecular
1160 Function, or Cellular Component ontology
1161

1162 **S3 Table.** List of primers used for cloning, PCR, RT-qPCR and qPCR

1163

1164 **S4 Table.** Expression of kanamycin resistance in the T1 generation of transgenic

1165 Arabidopsis plants.

1166

1167 **S1 Video/Movie** -Vesicle-associated localization of NISP

1168

1169 **S1 Fig. Identification of MP- and NSP-interacting proteins.**

1170 *In vitro* synthesized 3xHA-NSP and 3xHA-MP. In vitro translated HA-NSP and HA-MP were

1171 electrophoresed by SDS-PAGE and immunoblotted using an anti-HA antibody. **(B)** Protein

1172 interaction between *in situ* synthesized Arabidopsis hallo-proteins and 3xHA-MP or 3xHA-NSP.

1173 The AtNAPPA02 high-density array containing 4600 Arabidopsis ORFS spotted in duplicates was

1174 probed with 3xHA-MP or 3xHA-NSP, and candidate interactors were detected with an anti-HA

1175 antibody. The bright spots are signals from candidate interactors.

1176

1177 **S2 Fig. A network of NSP- and MP-directly interacting Arabidopsis proteins.**

1178 The network was assembled by the Cytoscape software. The viral proteins are indicated in red,

1179 MP-specifically interacting proteins in yellow, NSP-specifically interacting proteins in orange and

1180 proteins that associate with both MP and NSP are indicated in green.

1181

1182 **S3 Fig. *In silico* analyses of NISP.**

1183 **(A)** Phylogenetic tree of syntaxin superfamily domain-containing proteins from the species
1184 indicated. The phylogenetic tree was constructed using Bayesian inference performed with
1185 MrBayes v3.2.2 and the mixed amino acid substitution model Wag. The syntaxin-6 domain-
1186 containing proteins from Arabidopsis are indicated in blue, and NISP (AT4G30240) was clustered
1187 with plant-specific syntaxin-6 proteins forming the red clade. **(B)** Phylogenetic tree of N-terminal
1188 Syntaxin-6 domain-containing proteins. NISP (AT4G30240) clusters together with plant-specific
1189 N-terminal syntaxin-6 proteins, which do not harbor a typical t-SNARE domain at the C-terminus.
1190 The syntaxin-6 domain proteins from Arabidopsis are in blue. The phylogenetic tree was
1191 constructed using Bayesian inference performed with MrBayes v3.2.2 and the mixed amino acid
1192 substitution model Jones.
1193

1194 **S4 Fig. Sequence alignment of NISP and AT2G18860**

1195 The N-terminal syntaxin-6 domain is indicated in red and the transmembrane segment in black.
1196 The sequence alignment was performed using CLUSTAL OMEGA. Identical amino acids are
1197 indicated with asterisks, highly conserved residues with (:) and lower conservation with (.).
1198

1199 **S5 Fig. Subcellular localization of AT2G18860**

1200 AT2G18860-GFP distribution in plasma membrane-associated vesicles. *N. benthamiana* leaves
1201 were infiltrated with *A. tumefaciens* carrying a DNA construct expressing AT2G18860-GFP under
1202 the control of 35S promoter. Confocal images were taken 36 h post-infiltration. Approximately
1203 200 cells were examined. Scale bars, 10 μm.
1204

1205 **S6 Fig. *NISP*, but not AT2G18860, expression is induced by CabLCV**

1206 NISP and AT2G18860 transcript levels were quantified by qRT-PCR in uninfected Col-0 leaves
1207 (Col-0), tungsten-inoculated Col-0 leaves (Col-0 T), and systemically infected Col-0 leaves with
1208 CabLCV (Col-0 IN). Gene expression was calculated using the $2^{-\Delta Ct}$ method, and actin was used
1209 as an endogenous control. Error bars indicate 95% confidence intervals based on replicated
1210 samples (n = 3) from three independent experiments.
1211
1212 **S7 Fig. CabLCV infection-associated symptoms in Arabidopsis genotypes at 21 days post-**
1213 **inoculation (dpi).** The figure shows representative samples of mock-inoculated and CabLCV -
1214 infected plants. The genotypes are indicated in the figure.
1215
1216 **S8 Fig. The transgenic lines are visibly indistinguishable from the wild-type plants.**
1217 Columbia (Col-0) ecotype of *Arabidopsis thaliana* was used as the wild-type control for phenotype
1218 comparison, and the Col-0 ecotype was used for generating almost all the transgenic plants except
1219 for the complementation test in *nik1-1* and *at2g18860-1* mutants. The genotypes are the same as
1220 in Fig. 5. Plants were grown in a growth chamber at 22°C under long-day conditions (16 h light/8
1221 h dark). **(A)** Developmental phenotypes associated with inactivation of NISP gene and
1222 overexpression of NISP-GFP in the R3 generation of transgenic lines at 45 days after germination.
1223 **(B)** Developmental phenotypes associated with inactivation of NISP gene and overexpression of
1224 NISP-GFP in the R3 generation of transgenic lines at 60 days after germination. **(C)**
1225 Developmental phenotypes associated with inactivation of AT2G18860 gene and overexpression
1226 of AT2G18860-GFP in the R2 generation of transgenic lines at 45 days after germination. **(D)**
1227 Developmental phenotypes associated with inactivation of AT2G18860 gene and overexpression
1228 of AT2G18860-GFP in the R2 generation of transgenic lines at 60 days after germination

1229

1230 **S9 Fig. NSP enhances NISP-NIG complex formation.**

1231 **(A)** NISP interacts with NIG in yeast. AD-NIG and BD-NISP fusions were expressed in yeast and
1232 interactions between the recombinant proteins were monitored by His prototrophy in selective
1233 medium (-Leu,-Trp,-His) and supplemented with 2.5 mM 3-AT. The co-expression of PRNIG
1234 fused to BD and CSN5A fused to AD was used as a positive control. **(B)** Cytosolic and perinuclear
1235 distribution of NIG-GFP. *N. benthamiana* leaves were infiltrated with A. tumefaciens carrying
1236 NIG-GFP constructs with expression driven by the 35S constitutive promoter. NIG-GFP was
1237 imaged by confocal microscopy 36 h after infiltration. **(C)** Cytosolic localization of NIG in
1238 transgenic lines. Confocal fluorescence image of Arabidopsis root cells stably transformed with
1239 35S:NIG-GFP. **(D)** NISP interacts with NIG *in planta*. Total protein extracts from *N. benthamiana*
1240 expressing NISP-6HA and NIG-GFP were used for co-immunoprecipitation assays using anti-
1241 GFP. Input and IP show the levels of the expressed proteins NISP-6HA and NIG-GFP. Anti-HA
1242 was used to detect NISP-6HA from the immunoprecipitated complex. GFP was used as an
1243 unrelated protein. The experiment was repeated three times. **(E)** NISP-NIG complex formation in
1244 the presence of viral NSP. The Co-IP assay was performed as described in A, except that co-
1245 expressed NSP-GST was included in the assay. NSP-GST was detected by immunoprecipitating
1246 it from transfected leaves and immunoblotting with anti-GST. The experiment was repeated three
1247 times. **(F)** The interaction of NISP and NIG is increased by the presence of viral NSP. NIG-GFP
1248 levels in the immunoprecipitated complex in the presence and absence of viral NSP were
1249 quantified using the Band Analysis tools of the ImageLab software (Bio-Rad). The signal values
1250 were normalized using the IP NIG-GFP band, revealed in the same blot as the Co-IP band. A.U.
1251 denotes arbitrary unit.

1252

1253 **S10 Fig. NISP interacts with NIG *in vivo* and NSP enhances NISP-NIG complex formation.**

1254 **(A)** BIFC assay showing the interaction between NISP and NIG in vesicles of *N. benthamiana* leaf

1255 cells. *N. benthamiana* leaves transiently expressing NISP and NIG fused to the C-terminus (cYFP)

1256 or N-terminus (nYFP) of YFP, were examined by confocal microscopy 3 days after infiltration.

1257 Scale bars, 10 µm and 20 µm. **(B)** Co-expression of NSP-6HA strengthens interaction between

1258 NIG and NISP. The BiFC assay was performed by agro-infiltration of *N. benthamiana* leaves with

1259 nYFP-NIG, cYFP-NISP and vice-versus along with the expression of NSP-6HA. The reconstituted

1260 fluorescence signal was observed by confocal microscopy, 3 days after agroinfiltration. The figure

1261 displays representative samples from three independent biological repeats. Scale bars, 10 µm. **(C)**

1262 Confocal fluorescent image of NISP and NIG fused to the C-terminus (cYFP) or N-terminus

1263 (nYFP) of YFP in combination with empty vectors, as indicated in the figure.

1264

1265 **S11 Fig. Schematic representation of CabLCV DNA-A, DNA-B and ChIP-DNA primers.**

1266 **(A)** Schematic representation of DNA-A. The number 1 corresponds to the 5' end of the nick site

1267 (TAATATT/AC) within the conserved nonanucleotide sequence located in the intergenic region.

1268 The ORFs are indicated with arrows. The positions of the primers used for PCR (F1/R1), and qRT-

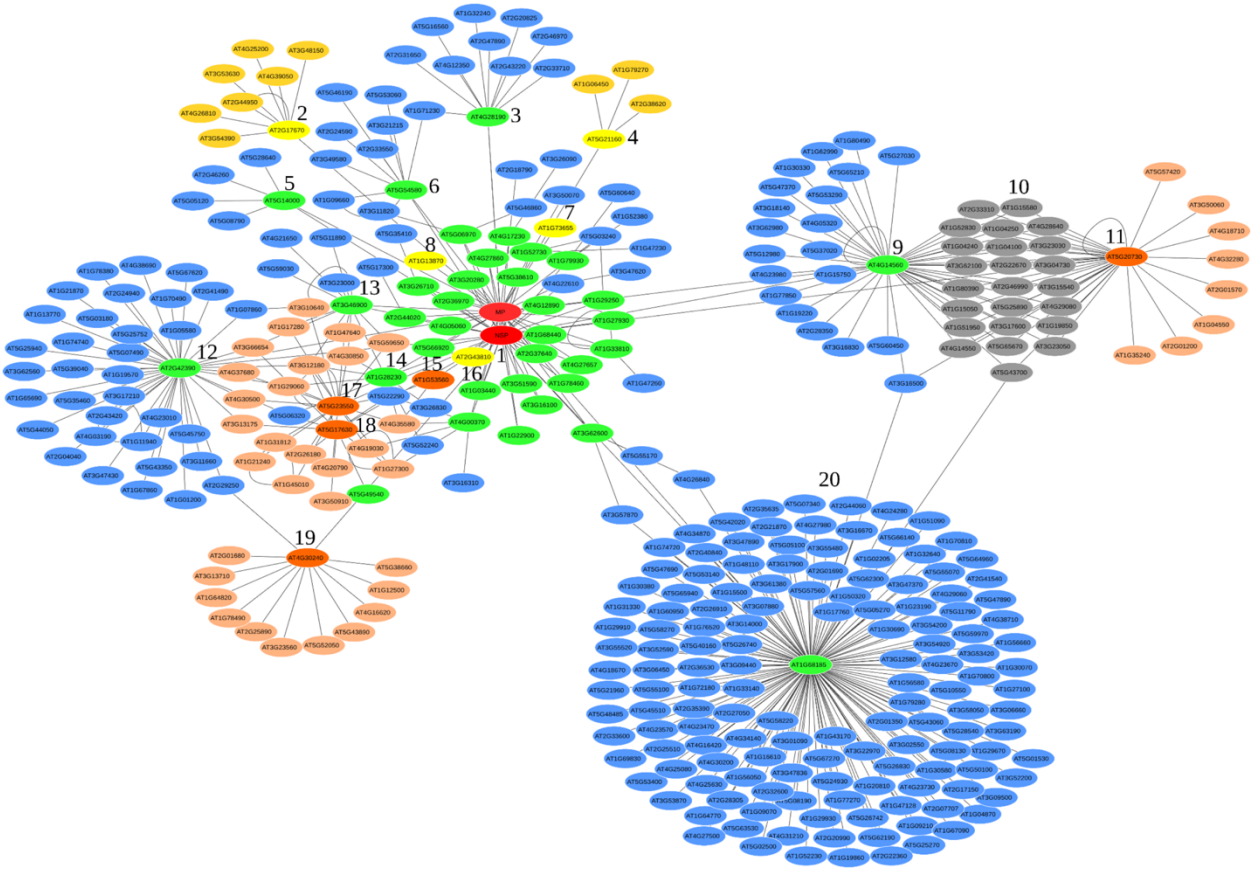
1269 PCR (F2/R2) are indicated. **(B)** Schematic representation of DNA-B. The numbering scheme is

1270 the same as in a. The ORFs are indicated with arrows. The positions of the primers used for PCR

1271 (F1/R1), and qRT-PCR (F2/R2 and F3/R3) are indicated.

1272

Figure 1



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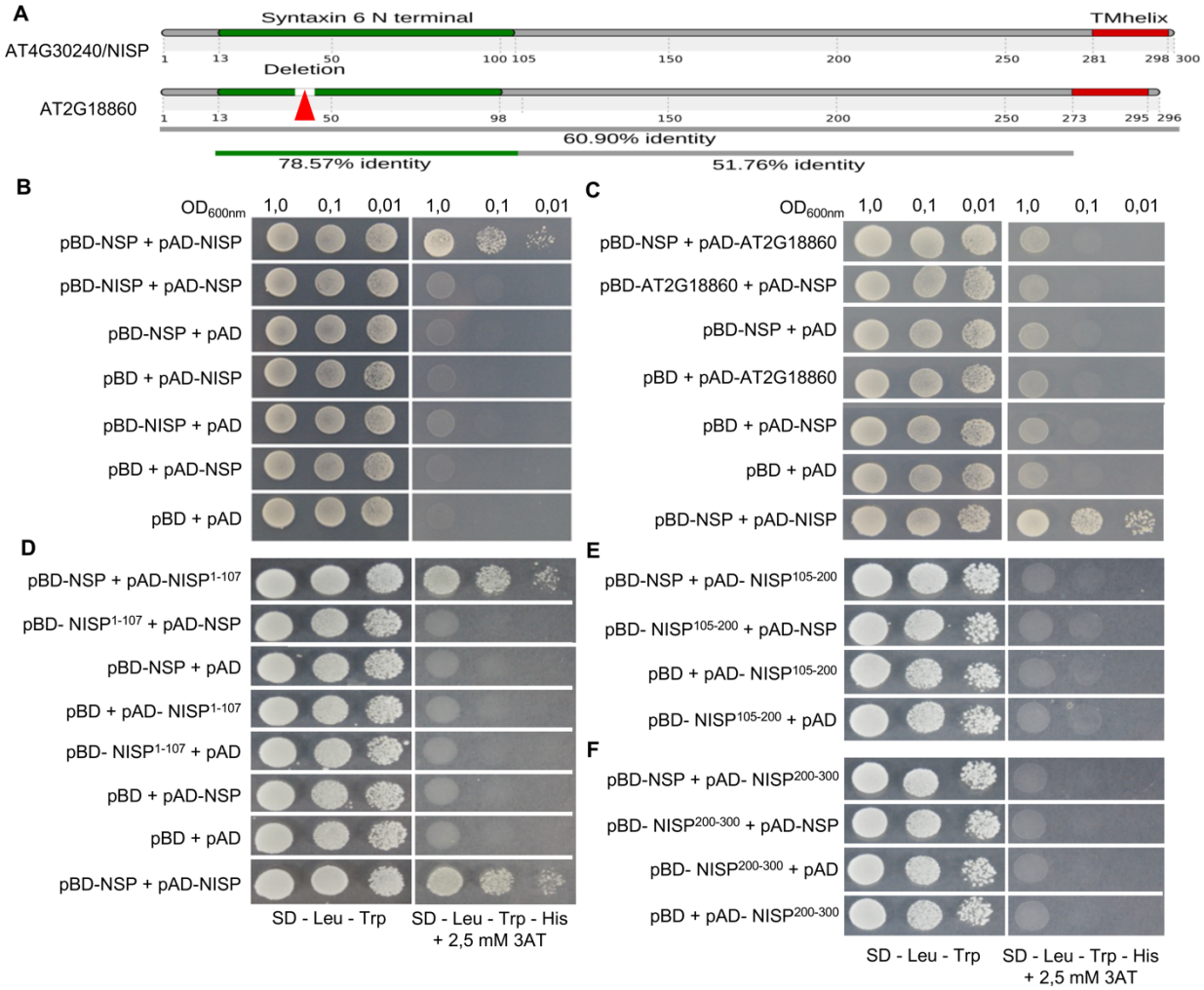

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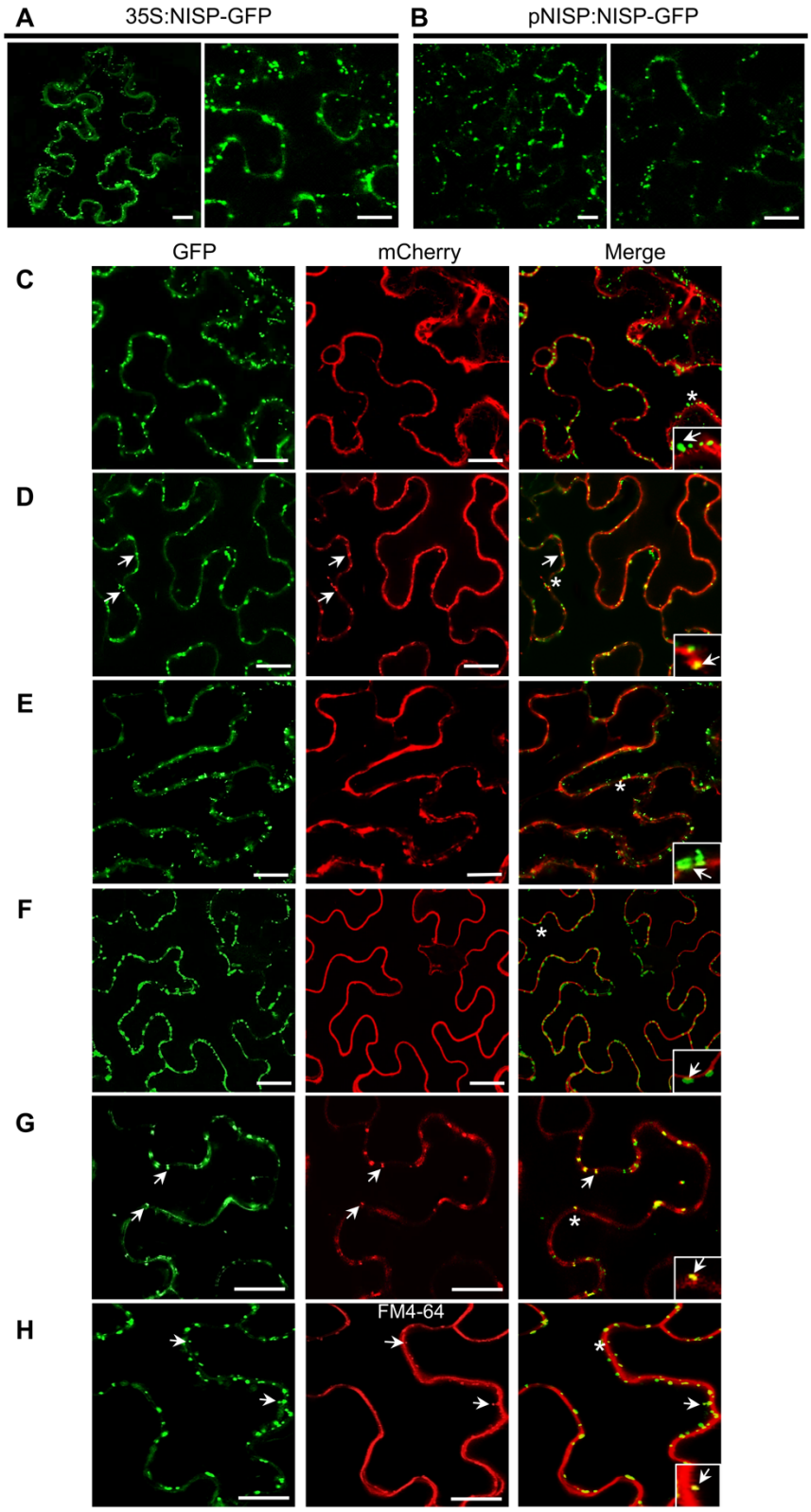

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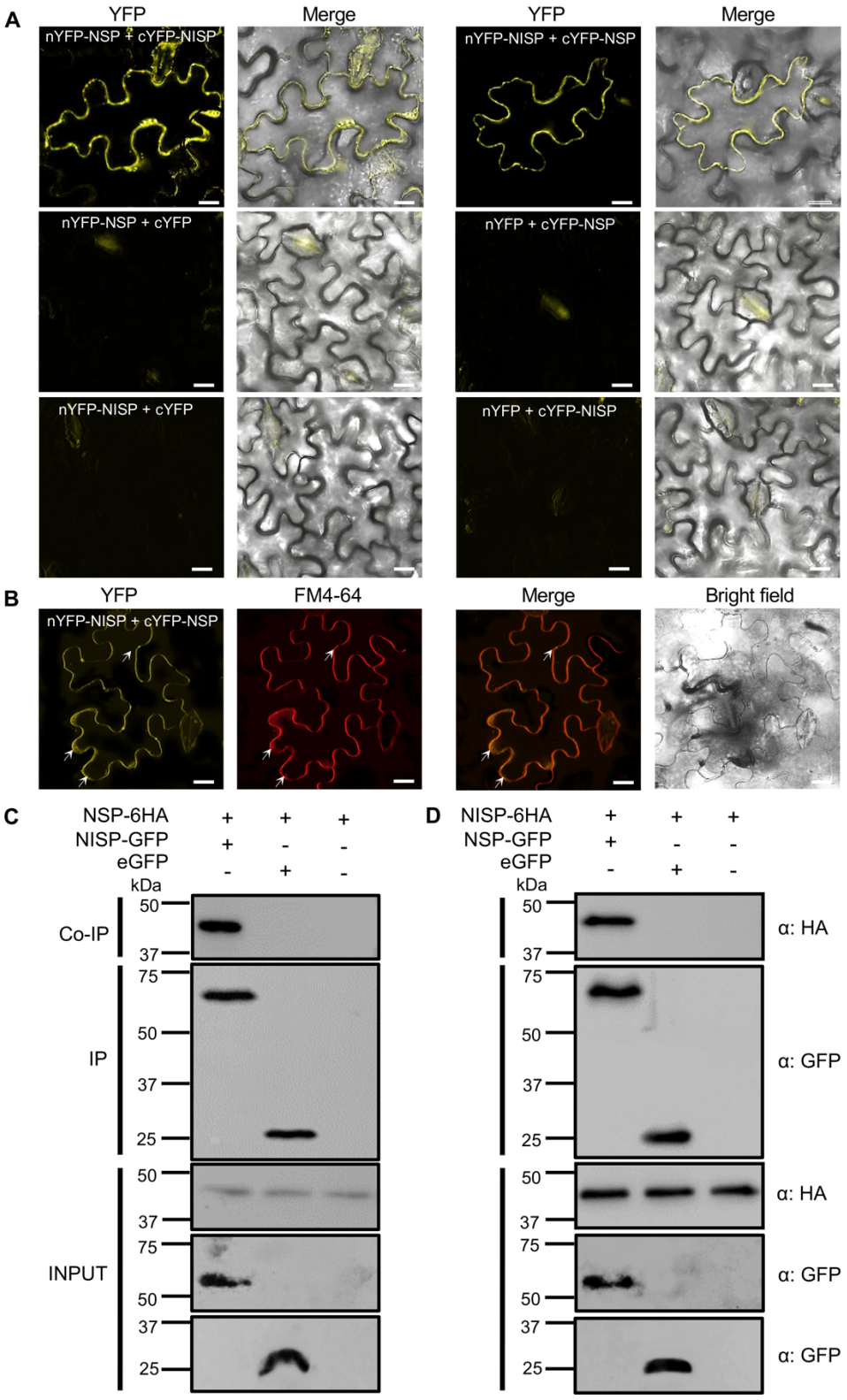

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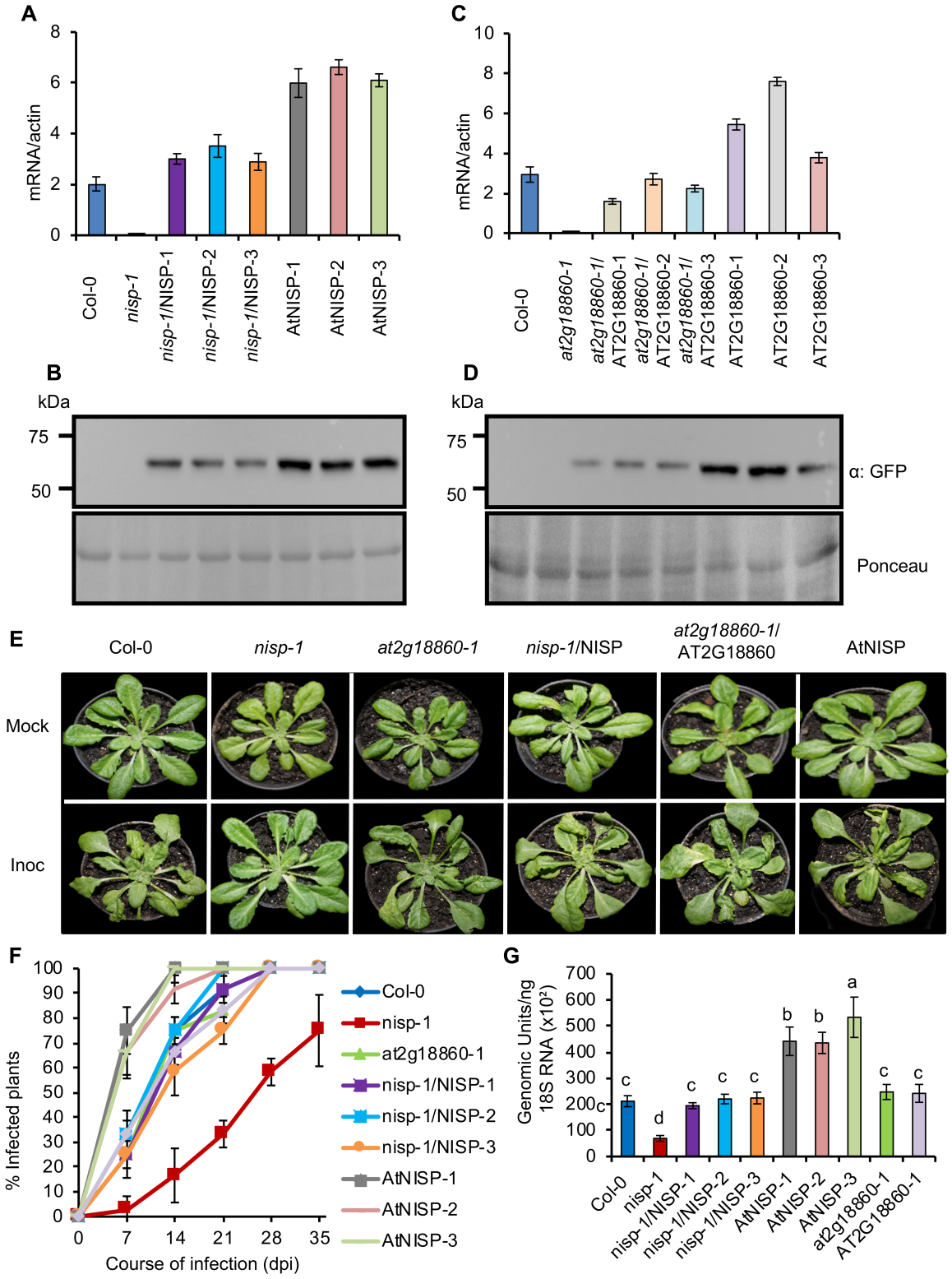

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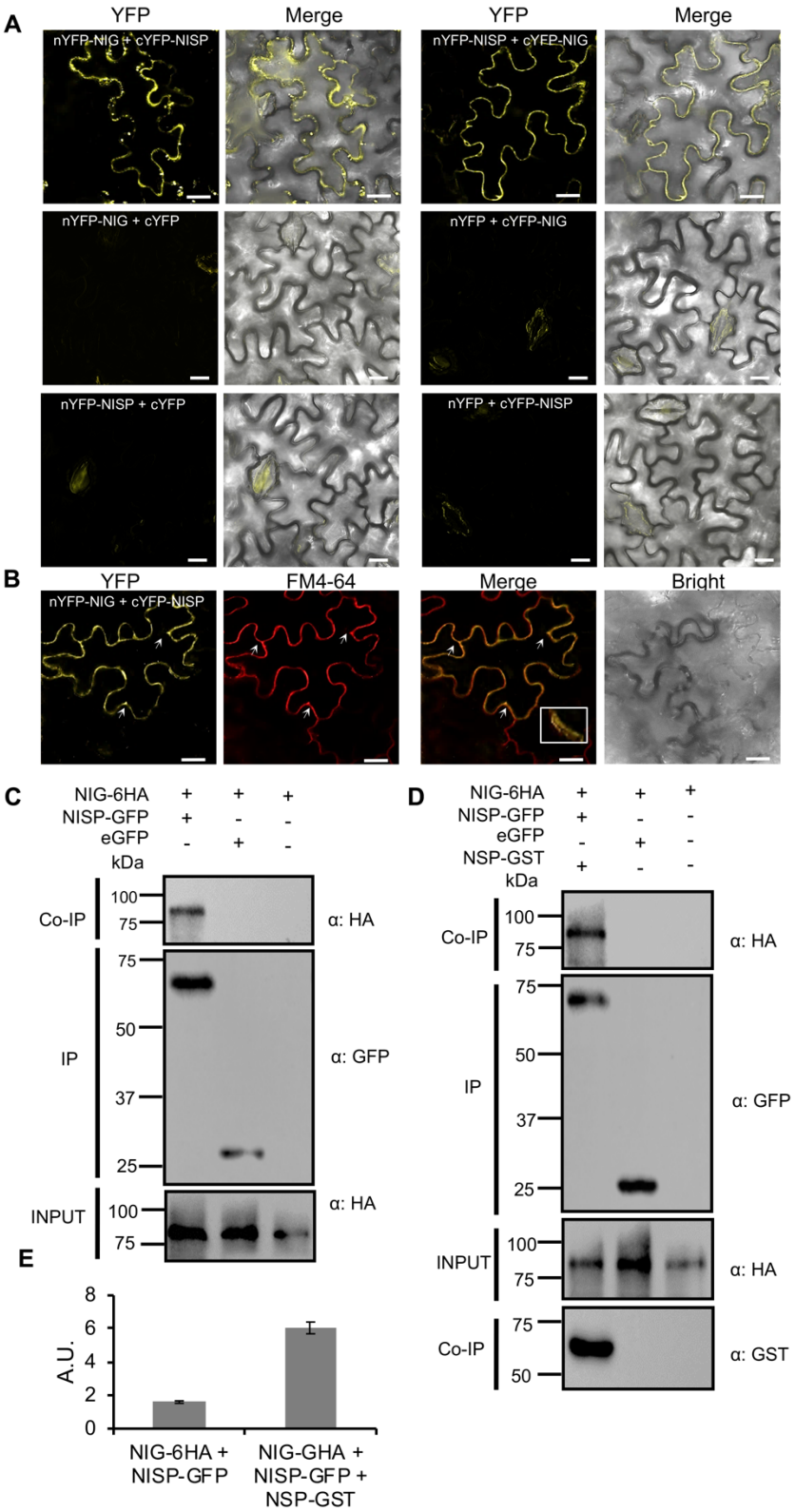
1283 Figure 6



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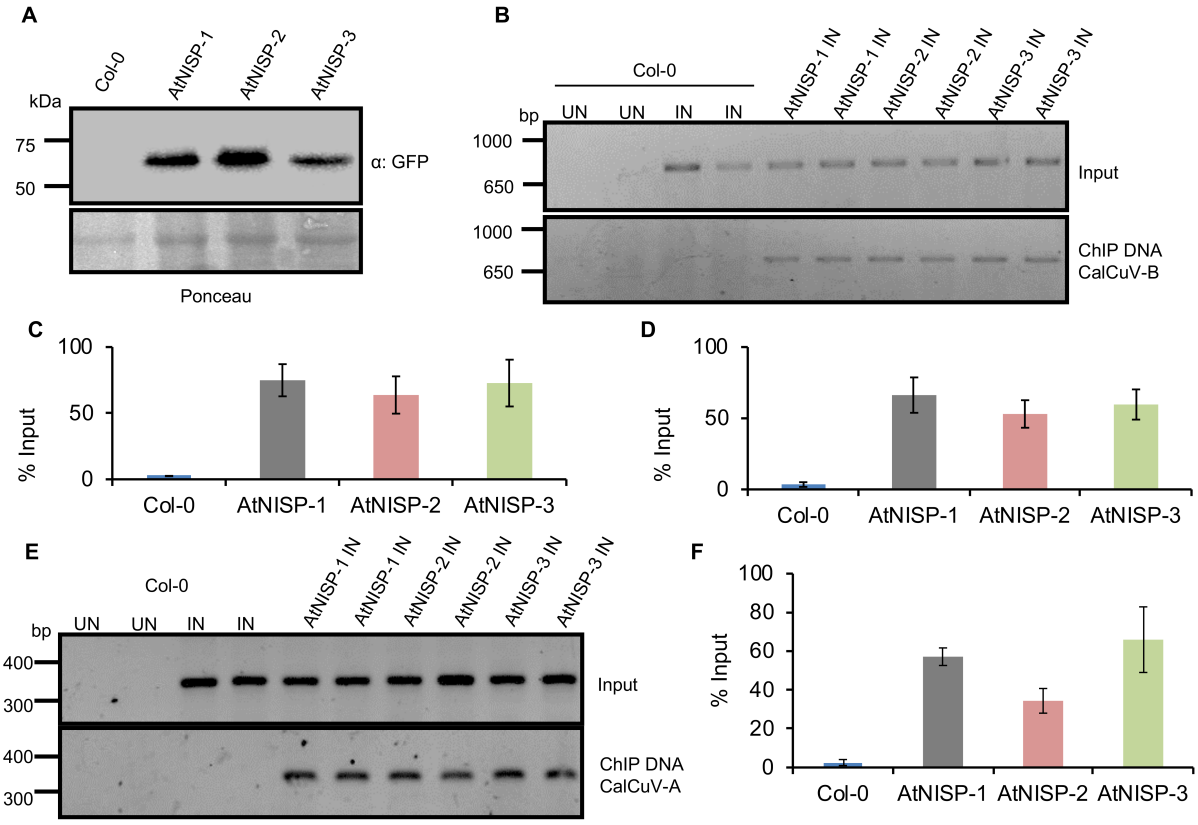
1285 Figure 7



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