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**Patellin 3 and 6, two members of the Plant Patellin family, interact with the movement protein of *Alfalfa mosaic virus* and interfere with the viral accumulation.**

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Running Title: *Interaction of AMV MP with Patellins*

## SUMMARY

Movement proteins (MP) encoded by plant viruses interact with host proteins to facilitate or interfere intra- and/or intercellular viral movement. Here, by using yeast two hybrid assays and bimolecular fluorescence complementation we present *in vivo* evidences of the interaction between the *Alfalfa mosaic virus* (AMV) MP and Arabidopsis Patellin 3 (atPATL3) and 6 (atPATL6), two proteins that contain a SEC14 domain. Proteins with SEC 14 domains are implicated in membrane trafficking, cytoskeleton dynamics, lipid metabolism and lipid-mediated regulatory functions. Interestingly, over-expression of either atPATL3 and/or 6 interfered with the MP to reach plasmodesmata resulting in a reduction of the size of the infection foci. Consistently, viral RNAs accumulation levels increased in the single and double Arabidopsis knockout mutants for atPATL3 and/or 6. Overall, our results indicate that this MP-PATLs interaction would interfere with the correct subcellular target of the MP making the intracellular transport of the viral MP containing-complexes less efficient and thus diminishing cell-to-cell movement.

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## INTRODUCTION

To establish a systemic infection, plant viruses must traffic from initially infected cells to neighbouring cells through plasmodesmata (PDs) channels until they reach the vascular system (Fernandez-Calviño *et al.*, 2011; Pallas *et al.*, 2011). This intercellular movement is an active process requiring one or more viral encoded movement proteins (MP) that interact with other viral factors (genome and other viral proteins) and with host proteins, altering in some instances the plant physiology (Pallas and Garcia, 2011; Whitham and Wang, 2004). In the last years different approaches have permitted to identify host proteins that interact with several MPs of the 30K superfamily (Melcher, 2000) and affect the viral movement in some cases (reviewed in Whitham and Wang, 2004; Boevink and Oparka, 2005; Lucas, 2006). Thus, the *Tomato spotted wilt virus* MP interacts with a DnaJ-like protein (Soellick *et al.*, 2000) and with At-4/1, a protein showing homology to myosin and kinesin motor proteins proposed as a component of the PD transport machinery (von Bargen *et al.*, 2001; Paape *et al.*, 2006). The MP of *Tobacco mosaic virus* (TMV) interacts with cytoskeleton components like the microtubule-associated protein MPB2C (Kragler *et al.*, 2003), microtubule end-binding protein 1 (EB1) (Brandner *et al.*, 2008), and actin filaments (McLean *et al.*, 1995) and with cell-wall associated proteins such as pectin methylesterase (PME) (Chen *et al.*, 2000) and calreticulin (Chen *et al.*, 2005). Moreover, it also interacts with a protein kinase associated to the PD (Lee *et al.*, 2005), with a DnaJ-like protein (Shimizu *et al.*, 2009), with a plant ankyrin repeat-containing protein (ANK) (Ueki and Citovsky, 2011) and with synaptotagmin, a calcium sensor that regulates vesicle endo and exocytosis (Lewis and Lazarowitz, 2010). Interaction with ANK and PME positively contributes to the TMV intercellular and systemic movement, respectively. The interaction with ANK cause a decrease in callose deposition, whereas that PME

regulates the viral unloading from the phloem (Ueki and Citovsky, 2011; Chen and Citovsky, 2003). Synaptotagmin is also required to the TMV systemic spread (Lewis and Lazarowitz, 2010). In contrast, calreticulin, MPB2C and EB1 negatively regulate the target of the TMV MP to the PDs (Kragler *et al.*, 2003; Chen *et al.*, 2005; Curin *et al.*, 2007; Brandner *et al.*, 2008). Additionally, it was described the interaction between the *Brome mosaic virus* MP and NbNaCa1, a protein that shows similarity to the  $\alpha$ -chain of nascent-polypeptide associated complex involved in the regulation of the MP localization in PDs (Kaido *et al.*, 2007). The MP of *Cauliflower mosaic virus* has been reported to interact with an Arabidopsis protein related to mammalian proteins described as rab acceptors (Huang *et al.*, 2001). *Tomato mosaic virus* MP has been shown to interact with putative transcriptional coactivators (KELP and MBF1) and protein kinases (Matshushita *et al.*, 2001, 2002, 2003; Yoshioka *et al.*, 2004). Overexpression of KELP interferes with the viral cell-to-cell movement (Sasaki *et al.*, 2009). By using a yeast-based approach it has recently found that the expression of *Prunus necrotic ringspot virus* MP triggers the GCN pathway through the activation of the Gcn2p kinase (Aparicio *et al.*, 2011).

AMV is the only member of the genus Alfamovirus in the family *Bromoviridae*. The AMV genome consists of three single-stranded RNAs of plus sense polarity. Replicase subunits P1 and P2 are encoded by the monocistronic RNAs 1 and 2, respectively, whereas RNA 3 encodes the MP and serves as a template for the synthesis of the non-replicating subgenomic RNA4 (sgRNA4) from which the coat protein (CP) is translated. AMV MP belongs to the 30K superfamily and is implicated in the intercellular viral movement (reviewed in Bol, 2005). Mutational analysis showed that the AMV MP has the ability to form tubular structures in protoplast which was correlated with the cell-to-cell movement capacity (Sanchez-Navarro and Bol, 2001).

However, identification of host factors interacting with AMV MP has not been reported until now.

In the present work we report the interaction between the AMV MP and two members of the Arabidopsis Patellin (PATLs) family: Patellin 3 and 6 (atPATL3 and atPATL6). PATLs are related to Sec14p (Peterman et al., 2004), which is the defining member of a family of phosphatidylinositol transfer proteins (Allen-Baume et al., 2002). Proteins related to Sec14p play a role in lipid signalling and metabolism and in membrane trafficking (Routt and Bankaitis, 2004). Biochemical fractioning and intracellular localization experiments showed that Patellin 1 (atPATL1) from Arabidopsis and zucchini (*Cucurbita pepo*) are peripheral membrane-associated proteins suggesting that PATLs would be implicated in vesicle/membrane trafficking events (Peterman et al., 2004, 2006). In fact, atPATL1 is critical in the formation and maturation of the cell plate during late telophase in Arabidopsis cells root (Peterman et al., 2004). Our subcellular localization analysis of the AMV MP in presence of atPATL3 or 6 indicate that these host proteins would interfere with the PD targeting of the MP diminishing the cell-to-cell movement. Accordingly, we found that transient over-expression of either atPATL3 and/or 6 reduced the size of the infection foci whereas viral RNAs accumulation levels increased in the single and double Arabidopsis knockout mutants for atPATL3 and/or 6.

## **RESULTS**

### ***AMV MP interacts with Patellin 3 and 6 from Arabidopsis.***

In order to shed light on the molecular mechanism driving the intercellular movement of the virus, we decided to search for host proteins that interact with AMV MP. Previous analysis addressed to identify host proteins involved in AMV transport by

yeast two-hybrid screens (Y2H) with full-length MP as bait yielded inconclusive results (Zuidmeer-Jongejan, 2002). We reasoned that the hydrophobic domain (HD) characteristic of the 30K superfamily (Sanchez-Navarro and Pallás, 1997), which has been shown in some viruses to be implicated in MP membrane association (Fujiki *et al.*, 2006; Martinez-Gil *et al.*, 2009), probably would interfere in the protein-protein interactions screened by the conventional GAL 4- based Y2H system. Thus, we decided to use a deleted version of the AMV MP lacking the HD as bait to screen a cDNA library of mRNA from *Arabidopsis thaliana* leaves by a GAL 4 based Y2H system (MATCHMAKER Two-Hybrid System 3, Clontech). From  $3 \times 10^6$  yeast transformants, we identified diverse potential interacting partners (our unpublished results). These included a deleted versions of the atPATL3 (at1g72160) (three clones) and atPATL6 (at3g51670) (two clones) lacking the N-terminal 285 or 210 residues, respectively (Supplemental Fig. 1A, ct-atPATL3 and 6). Attempts to corroborate these interactions with the full-length MP, by using the same Y2H system, showed that the viral protein interacted with ct-atPATL6 and more weakly with ct-atPATL3 but not with the full-length atPATL3 (Supplemental Fig. 1B). In order to confirm the interactions with the entire proteins we decided to use an alternative split-protein sensor system, which was specially designed to detect interactions between putative membrane-associated proteins. In this system the two interacting partners are expressed as fusion proteins with the N- and C-terminal fragments of the ( $\beta/\alpha$ )<sub>8</sub>-barrel enzyme N-(5-phosphoribosyl)- anthranilate isomerase (Trp1p) from *Saccharomyces cerevisiae*. The interaction between both fused proteins reconstitutes Trp1 activity permitting yeast cells to grow on medium lacking tryptophan (Tafelmeyer *et al.*, 2004). For this purpose, the N-terminal Trp1 fragment (NTrp) was fused to the N-terminus of the full-length atPATL3 or 6 to create NTrp:PATL3 and NTrp:PATL6, whereas that C-terminal Trp1

fragment (CTrp) was fused to the C-terminus of the full-length AMV MP resulting MP:CTrp (see Fig. 1). Plasmids were co-transformed into yeast cells and positive transformants were selected after incubation at 28°C for 3 days on minimal synthetic medium with tryptophan (SD+AHW). Positive protein interactions were detected under the same growth conditions but with minimal synthetic medium lacking tryptophan (SD+AH). As shown in Fig. 1, yeast cells co-transformed with MP:CTrp1 and NTrp:PATL3 or NTrp:PATL6 growth in interaction selective medium (SD+HA), whereas no growth was observed in the negative interaction controls: NTrp:PATL3 or NTrp:PATL6 co-transformed with the protein p53 (p53:CTrp) and MP:CTrp plus NTrp:eCFP (this plasmid express the the NTrp1 fragment fused to de N-terminus of the cyan fluorescent protein, eCFP).

Next, we used bimolecular complementation analysis (BiFC) as a second approach to corroborate these interactions *in planta* (Hu *et al.*, 2002; Aparicio *et al.*, 2006). Thus, the N-terminal fragment of the yellow fluorescent protein (YFP) (NtYFP) was fused to the C-terminus of AMV MP, whereas that the C-terminal fragment of the YFP (CtYFP) was fused to the N-terminus of atPATL3 and atPATL6 (see Fig. 2A). *Agrobacterium* mixtures of pMP-NtYFP plus pCtYFP-PATL3 or pCtYFP-PATL6 cultures were infiltrated in *Nicotiana benthamiana* leaves and fluorescence in epidermal cells was monitored by confocal laser-scanning microscopy (CLMS) at 2 days post-infiltration. We found that the reconstituted fluorescence was detected at the cell periphery and in punctate structures resembling PDs when the MP was co-expressed either with atPATL3 or atPATL6 (Fig. 2B, panels a and b). No fluorescence was detected neither when atPATL3 and 6 were co-infiltrated with the free NtYFP fragment nor when the MP was co-expressed with pCtYFP:ct38MPp (Supplemental Fig. 2, panels a, b and c; pCtYFP:ct38MPp express the CtYFP fragment fused to the last 38 residues



of the PNRSV MP; Aparicio et al., 2010). In Arabidopsis, Patellin family comprises six members characterized by a variable N-terminal domain followed by a Sec14 lipid-binding domain and a C-terminal GOLD domain (Fig 2A) (Peterman *et al.*, 2004). Since the GOLD domain is believed to be implicated in protein-protein interactions (Anantharaman and Aravind, 2002), we studied by BiFC technique whether the GOLD domain of the atPATL3 is involved in the interaction with the AMV MP. The GOLD domain of atPATL3 comprises amino acids from 353 to 487 (Fig. 2A) (see <http://www.uniprot.org/uniprot/Q56Z59>). Therefore, the CtYFP fragment was fused to the N-terminal region of the atPATL3 lacking the GOLD domain (CtYFP:NtatPATL3), or to the GOLD domain alone (CtYFP:GOLDatPATL3) (see Fig. 2A). Every construct was co-infiltrated joint the MP:NtYFP, and unexpectedly the fluorescence was reconstituted with CtYFP:NtatPATL3 but not with CtYFP:GOLDatPATL3 (Fig 2B, panels c and de, respectively). Western blot analysis confirmed that all fusion proteins were expressed properly (Fig. 2C). Overall, these results indicate that atPATL3 and 6 interact *in planta* with the AMV MP confirming the interactions by the Y2H system. Moreover, the GOLD domain is not strictly required to establish the interaction between MP and atPATL3 in the BiFC assay.

Previously, it was shown that the AMV MP accumulates at PDs (van der Wel et al., 1998). In fact, agro-expression of the MP fused to the green fluorescent protein (GFP) (MP:GFP) in *N. benthamiana* leaves showed a punctate structure pattern over the cell wall which represents PDs filled with MP:GFP (see Sánchez-Navarro and Bol, 2001; Herranz *et al.*, 2005; Oparka *et al.*, 1997). In this work we co-expressed the AMV MP:GFP and the MP of *Tobacco mosaic virus* fused to the red fluorescent protein (DsRed), a well-established PD marker (Roberts *et al.*, 2001; Crawford and Zambryski, 2001; Boiko *et al.*, 2000; Lucas, 2006), As expected, both proteins co-localize in PDs

(Supplemental Fig. 3A). As stated above the BiFC analysis showed that a pool of the MP-atPATLs complexes locate in PDs. This observation opens the question whether atPATL3 and 6 accumulate at these structures. Previous studies reported that PATL1 seems to be a cytoplasmic protein that can associate with cellular membranes (Peterman et al., 2004, 2006). Then, we analyzed the subcellular localization of atPATL3 and 6 by transient agroexpression of the proteins fused with the GFP at its C-terminus (atPATL3:GFP and atPATL6:GFP) . Images by CMLS showed that the both fusion proteins accumulated at the cellular periphery (Supplemental Fig 3B, panels a and c). Nevertheless, despite that both atPATL:GFP strongly mark the cell periphery it seems they do not accumulate at PDs. (Supplemental Fig. 3B panels b and d). In summary, the punctate pattern observed by the BiFC assay represents PDs filled with MP/atPATL complexes. Furthermore, the fact that a pool of atPATL3 and 6 changes its subcellular localization as a result of the interaction with the viral protein suggest that the MP would be interacting with these atPATLs somewhere out of the PDs and that the MP/atPATL complexes are transported towards the PD.

### ***MP-atPATLs interaction interferes with the viral infection***

Next step was to examine whether atPATLs-MP interactions could have an effect on the viral infection. Firstly, we tested the effect of the over-expression of atPATL3 and atPATL6. For this purpose, two leaves from three plants per construct of transgenic *Nicotiana tabacum* plants expressing the AMV P1 and P2 proteins (P12 plants, Taschner *et al.*, 1991) were inoculated with RNA transcripts from a modified AMV RNA 3 clone that express the GFP together with the MP and the CP (Sanchez-Navarro *et al.*, 2001) (Fig. 3A). This construct permits to visualize infection foci and measure their area (Figure 3B). At 24 hours post-inoculation leaves were co-infiltrated

at 0.4 OD with binary plasmids expressing atPATL3, atPATL6, a mixture of both (atPATL3-6) or the luciferase (LUC) as negative control.

By fluorescence microscopy, images of 50 individual infection foci/leaf were acquired at 4 days post-inoculation (dpi) and their area was measured using Image J software (Wayne, Rasband, National Institutes of Health, Bethesda, MD, USA; <http://rsbweb.nih.gov/ij>). Foci were grouped into three different categories attending their area size. As shows graph in Fig. 3C the percentage of fluorescent foci with an area smaller than 2 mm<sup>2</sup> was 25% in control LUC infiltrated leaves whereas it rose until 42%, 52% and 67% in the corresponding atPATL3, atPATL6 and atPATL3-6 infiltrated leaves, respectively. In contrast, the percentage of foci with an area higher than 3 mm<sup>2</sup> was 46% for LUC leaves but it decreased until 29%, 4% and 14% in the different atPATLs infiltrated leaves. This experiment was repeated two times with similar result. Overall, these results indicated that over-expression of both atPATLs diminished the cell-to-cell movement.

The decreased foci size could be caused by a reduction of the cell-to-cell movement capacity as a consequence of an impaired targeting of the MP to the PDs. In order to determine if the AMV MP sub-cellular localization was affected by the over-expression of atPATLs, we compared the distribution pattern of MP:GFP alone or co-agroinfiltrated with atPATL3 or 6. In cells expressing the MP:GFP alone the protein was exclusively localized at PDs (Fig. 4, upper panels). In contrast, when any of the two atPATLs was co-expressed with MP:GFP, we found that in most cells the fluorescence was located in addition to PDs, in spherical inclusion bodies. Moreover, at higher magnification irregular filamentous structures could also be observed (Fig. 4, middle and lower panels, respectively. Only the effect with atPATL3 is shown). These results suggest that atPATLs are interfering with the PD localization of a pool of the MP.

Finally, we analyzed how the absence of atPATL3 and 6 affected the viral accumulation. Thus, Arabidopsis T-DNA insertion mutants for atPATL3 (*atpatl3*, SALK093994), atPATL6 (*atpatl6*, SAIL\_284\_B11) were isolated and the double mutant constructed (*atpatl3-6*). PCR analysis using atPATLs and T-DNA left border specific primers was carried out to verify the homozygosity of the mutants. RT-PCR analysis confirmed the absence of detectable atPATL3 and 6 mRNAs corroborating that mutations resulted in loss of expression (data not shown). Moreover, these mutants germinated in MS medium and growth in soil showing a similar phenotype to the wild type (wt) (Figure 5A). *A. thaliana* mutants and wt were inoculated with compatible AMV PV0196 isolate (DSMZ GmbH, Plant Virus Collection, Germany) virions. At 4 dpi total RNA extracted from inoculated leaves was analyzed by Northern blot to detect the accumulation of the viral RNA 3 and 4 using a Digoxigenin-labelled AMV CP probe. Northern blot in Fig. 5B shows RNA 3 and 4 accumulation levels of five independent plants from one experiment. Northern blot signal was quantified using Image J software (Fig 5C). The graph illustrates that AMV RNAs accumulated at higher levels in *atpatl3*, *atpatl6* and *atpatl3-6* than in wt plants. This experiment was repeated again with similar results. Overall our results indicate that the interaction between atPATL3 or 6 with MP negatively affects the AMV accumulation in Arabidopsis plants.

## DISCUSSION

The Arabidopsis Patellin family is composed by six members (designed as PATL1-6) characterized by showing a variable N-terminal region followed by a Sec14-like domain and a C-terminal GOLD domain (Peterman *et al.*, 2004). The yeast protein Sec14p is the prototype module known as the SEC14 domain which is a lipid binding domain. Proteins with Sec14 domain are involved in membrane trafficking,

cytoskeleton dynamics, lipid metabolism and lipid-mediated regulatory functions (reviewed in Philips *et al.*, 2006; Bankaitis *et al.*, 2007; Mousley *et al.*, 2007). In contrast, GOLD domains are characteristic in several proteins involved in Golgi function and vesicle traffic and they are presumed to act as protein-protein interaction domains (Anantharaman and Aravind, 2002). PATLs are distributed across the plant kingdom although very little is known about its *in vivo* functions. Database mining indicates that in Arabidopsis atPATL3 and atPATL6 are expressed at some degree in the whole plant, including roots, at the vegetative stage, in the entire rosette and internodes after plant transition to flowering and in flowers, siliques and seeds (Winter *et al.*, 2007). Biochemical and intracellular localization experiments carried out with PATL1 from Arabidopsis and zucchini reported that this protein binds phosphatidylinositol and exist in a cytoplasmic pool that can associate with cellular membranes playing a critical role in formation and maturation of the cell plate during late telophase in Arabidopsis cells roots (Peterman *et al.*, 2004; 2006). PATLs would interact with membranes through the Sec-14 domain acting as adaptors for recruiting GOLD-domain binding proteins to specific membrane sites. On the other hand, cell-to-cell traffic of viral genomes requires the interaction of the MPs with the host cytoskeleton components and endomembrane system to reach the PDs (reviewed in Boevink and Oparka, 2005; Lucas, 2006; Hofmann *et al.*, 2007).

We have shown that atPATL3 and 6 interact with the AMV MP in yeast and *in planta*. Moreover, BiFC results reproduce the typical punctuate accumulation pattern at the cell periphery of the viral protein suggesting that MP/atPATLs complexes accumulated at the PDs. The fact that at least atPATL3 does not accumulates at PDs when is expressed alone suggest that probably this pattern is a result of the interaction

with the MP which in its transport towards the PDs drags the atPATLs molecules modifying its subcellular localization.

Surprisingly, the BiFC analysis also showed that a deleted atPATL3 version lacking the GOLD domain (NtatPATL3) was still able to interact with the MP whereas the GOLD domain alone did not. Besides, the GAL 4-based Y2H system showed that a truncated version of atPATL3 and 6 containing the C-terminal part of the Sec14 and the entire GOLD domains were able to interact with the AMV MP (Supplemental Fig 1). Overall, these observations would indicate that the GOLD domain is not strictly necessary to establish the MP-atPATL3 interaction and that at least part of the Sec14 domain is required to occur this interaction. However, we cannot discard that the construct containing the GOLD domain alone could codify a protein with an inefficient folding not suitable to interact with the MP. The presence of part of the Sec14 C-terminal domain could permit to achieve the correct folding of the GOLD rendering a functional domain.

In addition to the accumulation of the MP/atPATL complexes at the PDs, we have also found that over-expression of atPATLs retains the MP in spherical and filamentous structures (Fig. 4). We cannot rule out the possibility that the abnormal high amount of these structures could be induced by an elevated level of MP and atPATLs, as consequence of the agro-infiltration expression procedures.

Over-expression and absence of the atPATLs induced an opposite effect on viral infection. Overall, our results show that both atPATL3 and 6 difficulty the cell-to-cell movement and reduce viral accumulation. In this sense, it is tempting to speculate that the interaction of AMV MP with atPATL3 or 6 would interfere with the intercellular movement of the viral MP containing-complexes anchoring them to cellular membranes and PDs, which negatively affects to the viral transport towards and through PDs. Thus,

PATLs would operate as a defensive barrier against AMV infection. Impairment in viral spread has been reported as a result of over-expression of different plant proteins which directly interact with viral MPs; in most cases this interference has been found to be associated with changes in the intracellular localization patterns of MPs (Kragler *et al.*, 2003; Chen *et al.*, 2005; Curin *et al.*, 2007; Brandner *et al.*, 2008; Sasaki *et al.*, 2009, see Pallas and Garcia, 2011 for a review). Future experiments will permit to unravel the mechanism by which PATLs interfere with AMV transport and subcellular localization.

## EXPERIMENTAL PROCEDURES

### Plasmid construction

The full-length ORFs of atPATL3 and 6 were amplified by RT-PCR from Arabidopsis total extracted RNA with specific sense and antisense primers, containing the restriction site *Sfi*I. The amplified fragments were exchanged by the eCFP (cyan fluorescent variant protein) gene in the pNtrp-eCFP plasmid digested with *Sfi*I and dephosphorylated. The resultant clones, pNtrp-atPATL3 and pNtrp-atPATL6, contain the 42 N-terminal amino acids of the ( $\beta/\alpha$ )<sub>8</sub>-barrel enzyme N-(5-phosphoribosyl)-anthranilate isomerase (Trp1p) from *Saccharomyces cerevisiae* fused at the N-terminus of the host proteins PATLs. The AMV MP gene was amplified from the AMV cDNA3 (Sánchez-Navarro et al., 2001) with specific primers containing the *Sfi*I sequence and inserted in the pI-Ctrp3 plasmid, previously digested with *Sfi*I and dephosphorylated. The resultant clone contains the 179 C-terminal amino acid of Trp1p protein fused at C-terminus of the MP.

A modified pSK plasmid (psk35S) containing the cauliflower mosaic virus 35S promoter followed by a multiple cloning site including *Nco*I and *Nhe*I restriction sites and the potato proteinase inhibitor terminator (Popit) was used to generate GFP and BiFC fusion proteins. Full-length GFP, NtYFP or CtYFP fragments were PCR-amplified with specific pairs of sense/antisense primers containing *Pag*I (compatible with *Nco*I) /*Nco*I restriction sites or *Nhe*I/ *Xba*I (compatible with *Nhe*I) restriction sites. PCR products were cloned into *Nco*I linearized psk35S plasmid to obtain psk35S:NtYFP-(*Nco*I-*Nhe*I)-Popit and psk35S:CtYFP-(*Nco*I-*Nhe*I)-Popit or into *Nhe*I linearized psk35S plasmid to obtain psk35S:(*Nco*I-*Nhe*I)-GFP-Popit, psk35S:(*Nco*I-*Nhe*I)-NtYFP-Popit and psk35S:(*Nco*I-*Nhe*I)-CtYFP-Popit. atPATL3, atPATL6,

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NatPATL3, and GOLDatPATL3 ORFs were PCR-amplified with specific sense and antisense primers containing a *NcoI* and *NheI* restriction sites respectively, and cloned either in psk35S:CtYFP-(*NcoI-NheI*)-Popit or psk35S:(*NcoI-NheI*)-GFP-Popt. AMV MP was PCR-amplified and cloned into psk35S:(*NcoI-NheI*)-NtYFP-NOS. Finally, the resultant psk35S:fusion proteins- cassettes were introduced into the *XhoI* digested pMOG800 binary vector. Plasmid px032/GFP-MP-CP has been described in Sánchez-Navarro et al., 2001. Binary plasmids expressing the AMV MP fused to the GFP (MP-GFP) and CtYFP:38aaMPp have been previously described (Herranz *et al.*, 2005 and Aparicio *et al.*, 2010, respectively).

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### Split-protein sensor yeast two-hybrid based system

pNtrp-PATL3 or pNtrp-PATL6 plasmid was co-transformed with pI-AMV MP-Ctrp3 clone into .....yeast ~~cells~~ strain auxotroph for showing Tryptophan (W), Histidine (H), Adenine (A), Uracile (URA3) and Leucine (L) ~~auxotrophies~~. Positive transformants were selected after incubation at 28°C for 3 days on minimal synthetic medium lacking without Uracile and Leucine (SD+AHW). Positive protein interactions were detected under the same growth conditions and minimal synthetic medium but withoutlacking tryptophan (SD+HA).

### Agroinfiltration and laser scanning confocal analysis

Binary plasmids were transformed into *Agrobacterium tumefaciens* C58 cells by ~~electroporation~~ and spread on LB plates containing 50  $\mu$ g/ml of kanamycin and 25  $\mu$ g/ml of rifampicin (LBkr). For BiFC analysis positive colonies were grown in liquid LBkr at 28 °C for 24 hours and the bacterial cultures were suspended in infiltration buffer (10 mM MgCl<sub>2</sub>, 10mM MES pH 5,6) at 0.4 OD600 into 3 week old *Nicotiana benthamiana* plants. The YFP expression was analyzed at 2 days post-infiltration with a

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Leica TCS SL confocal laser scanning microscope (Leica), (GFP excitation and emission at 488 and 535 nm, respectively).

### **Plant inoculation**

*Arabidopsis* or *N. tabacum* P12 plants were grown in pots in a growth chamber at 24°C with a photoperiod of 16 h light/8 h dark. Inoculation of *Arabidopsis* plants was carried out with purified virions of AMV PV0196 isolate (DSMZ GmbH, Plant Virus Collection, Germany). Three leaves of four week olds *Arabidopsis* plants were mechanically inoculated with 30 mM Sodium phosphate buffer pH 7. P12 plants were inoculated with transcripts of the modified RNA 3 expressing the GFP (GFP-MP-CP). Plasmid R3 GFP-MP-CP was linearized with *Pst*I and transcribed with T7 RNA polymerase (Roche) following the manufacturer's recommendations. Transcripts (5 ug/leaf) were mechanically inoculated on two leaves of two P12 plants.

### **Transient over-expression of Patellins in P12 plants**

At 24 hours post-inoculation P12 leaves were infiltrated with *Agrobacterium* cultures expressing the Luciferase protein, atPATL3 or 6 prepared in infiltration buffer at 0,4 OD600-. GFP expression of infection sites was visualized at 4 dpi with a LEICA MZ16F fluorescence stereomicroscope. The area of the foci was measured using Image J software (Wayne, Rasband, National Institutes of Health, Bethesda, MD, USA; <http://rsbweb.nih.gov/ij>)

### **Northern and western blots**

Inoculated leaves were harvested at indicated times. Leaves were ground in liquid nitrogen with mortar and pestle. Total RNA was extracted from 0.1 g leaf material using Trizol Reagent (Sigma). RNAs were denatured by formaldehyde treatment and analyzed by northern blot hybridization as described previously (Sambrook *et al.*, 1989). RNAs were visualized on blots using a Digoxigenin labelled

riboprobe corresponding to the AMV CP ORF. Sintesis of Digoxigenin labelled riboprobes, hybridization and Digoxigenin detection procedures were carried out as previously described (Pallas *et al.*, 1998).

For western blot analysis total proteins were extracted from 50 mg leaf tissue. Samples were homogenized with 100 µl of Laemmli buffer (Laemmli, 1970), boiled 3 min and 25 µl of extracts were resolved in a 14% SDS-PAGE. Gels were electrotransferred to PDVF membranes (Amersham) following the manufacturer's recommendations. Detection of NtYFP-fused proteins was carried out with anti-GFP N-terminal antibody (Sigma; Sant-Louis, Missouri; product number G1544) whereas CtYFP-fusion proteins were detected using an anti GFP antibody (Roche; Cat No 11814460001). Detection procedures were carried out following the manufacturer's recommendations.

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## FIGURE LEGENDS

**Fig. 1.** Trp1 yeast two hybrid assays. Different dilutions (on the top) of the co-transformed yeast cells with the indicated pair of plasmids (on the left) were spotted on synthetic minimal medium containing (SD+AHW) or lacking ~~lacking~~ Tryptophan (SD+HA) to confirm a proper transformation or a positive interaction, respectively. Self-interaction of MP (MP:NTrp + MP:CTrp) and interaction of NTrp:atPATL3 or NTrp:atPATL6 with p53:CTrp and MP:CTrp with NTrp:eGFP were used as positive and negative controls, respectively.

**Fig. 2. Analysis of the MP-PATLs interaction by the BiFC technique.** (A) Schematic representation of the clones expressed joint the AMV MP:NtYFP. The first two constructs represent the full-length atPATL3 and 6 showing the domain architecture: the characteristic threonine rich N-terminus, central Sec 14 and C-terminal GOLD domains. The next two constructs represents the deleted versions of atPATL3 lacking the GOLD domain or the threonine-rich and sec-14 domains, respectively. Numbers correspond to the amino acid residue positions. (B) BiFC analysis to identify the atPATL3 domains implicated in MP-PATL3 interaction. Images of epidermal *N. benthamiana* leaves captured by confocal laser scanning microscope. Leaves were infiltrated with MP:NtYFP joint (a) CtYFP:atPATL3, (b) CtYFP:atPATL6, (c) CtYFP:NtatPATL3 or (d) CtYFP:GOLDatPATL6. White arrows indicate reconstituted fluorescence in punctate bodies representing PDs. Bar = 10  $\mu$ m. (E) Western analysis of the expression of the corresponding fusion proteins analyzed in (B). The detection was made with

specific antibodies detecting NtYFP and CtYFP fusion proteins (Nt and Ct, panels, respectively) Lanes 1 to 4 leaves infiltrated with MP:NtYFP plus CtYFP:atPATL3, CYFP:atPATL6, CYFP:NatPATL3 or CYFP:GOLDatPATL3, respectively. Lane 5 non-infiltrated leaves. Asterisk denotes an unspecific host protein.

**Fig. 3.** Effect of the PATLs over-expression on viral infection (A) The schematic representation shows the AMV RNA 3 expressing the GFP (R3-GFP). The open reading frames corresponding to the green fluorescent protein (GFP), the movement protein (MP) and the coat protein (CP) are represented by big boxes. (B) Representative images of the foci size induced by the inoculation the RNA transcribed from the R3-GFP construct in *N. tabacum* P12 leaves infiltrated with binary plasmids expressing LUC or a mixture of atPATL3 plus 6. (C) Graph showing the percentage of foci grouped into three different ranges of area size. The foci area were measured in *N. tabacum* P12 plants, inoculated with transcripts of R3-GFP and agro-infiltrated with binary plasmids expressing atPATL3, atPATL6, a mixture of both (atPATL3-6) or the luciferase (LUC) as negative control.

**Fig. 4.** Confocal laser scanning microscope images of epidermal *N. benthamiana* leaves infiltrated with the *Agrobacterium* cultures expressing the proteins indicated on the left. Upper panels shows punctuated bodies representing PDs filled with MP:GFP. Middle and lower panels show the spherical inclusion bodies and cytoplasmic irregular filamentous structures, respectively, observed when the MP:GFP was co-expressed with atPATL3 (denoted by white arrows).

**Fig. 5.** Study of the viral accumulation in Arabidopsis knockouts for atPATLs (A) Photograph of 10-days-old seedlings of the Arabidopsis wild type (wt) and knockouts. Seedlings were grown on agar plates with MS medium under controlled growth conditions. (B) Detection of AMV accumulation in Arabidopsis wt and knockout lines by Northern blot analysis of five infected plants. RNA 3 and 4 are indicated on the left. Lower panel is the Ethidium bromide (EtBr) stained gel as loading control. (C) Graph showing the average of viral RNAs accumulation measured from the Northern blot in B. Standard deviation values are shown. Significant differences are indicated by \* $P < 0.05$ .

**Supplemental Fig. 1.** Study of the MP-PATLs interaction by the conventional GAL 4-based Y2H system (A) Schematic representation of the full-length Arabidopsis atPATL3 showing the domain architecture of the protein (see [www.uniprot.org/uniprot/Q56Z59](http://www.uniprot.org/uniprot/Q56Z59)). pAD:ct-atPATL3 and pAD:ct-atPATL6 represent the deleted versions of atPATL3 and atPATL6 lacking the N-terminal 285 or 210 amino acids, respectively, found as interacting partners of the AMV MP (B) Co-transformed yeast cells (AH109) with the pBD:MP or empty pBD (pGBKT7) plasmids and pAD:atPATL3, pAD:ct-atPATL3 and 6 were spotted on minimal synthetic dropout (SD) medium containing (SD+HA) or lacking (SD) histidine and adenine to confirm a proper co-transformation or a positive interaction, respectively. Cells were grown at 28°C for 4 days. Interaction with the empty pBD vector was used as negative control.

**Supplemental Fig. 2.** Negative controls used in the BiFC analysis. *N. benthamiana* leaves were co-infiltrated with the NtYFP fragment plus CtYFP: PATL3 or CtYFP: PATL6 (panel a and b, respectively) and MP: NtYFP plus CtYFP.38aaMPp (panel c). MP-NtYFP plus CtYFP: atPATL3 was used as positive interaction (panel d).

In all cases agrobacterium cultures were infiltrated at 0.5 OD600 and images were collected at 2 days post-infiltration by CLSM (GFP excitation and emission at 488 and 535 nm respectively).

**Supplemental Fig. 3.** Subcellular localization AMV MP, atPATL3 and atPATL6. (A) Co-localization at plasmodesmata of the AMV and TMV MPs. Leaves of *N. benthamiana* plants were co-infiltrated with agrobacterium cultures carrying binary vectors that express the MPs of AMV and TMV fused to the GFP (MP-GFP) and the dsRed (MPTMV-dsRed), respectively at 0.2 OD600. Images were collected at 2 days post-inoculation by CLSM (GFP excitation and emission at 488 and 535 nm, respectively; DsRed excitation and emission at 557 nm and 579 nm, respectively). (B) Subcellular localization of atPATL3 and 6 with the GFP was fused at their C-terminus (atPATL3:GFP and atPATL6:GFP) and infiltrated in *N. benthamiana* leaves. CLSM images shows that the proteins present a strong signal at the cell periphery (panels a and c). Magnification of the cell periphery shows that both, atPATL3:GFP and atPATL6:GFP do not seem to accumulate at PDs (panels b and c). In all cases agrobacterium cultures were infiltrated at 0.2 OD600 and images were collected at 2 days post-inoculation (GFP excitation and emission at 488 and 535 nm, respectively).