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The coat protein of Alfalfa mosaic virus interacts and interferes with the transcriptional activity of the bHLH transcription factor ILR3 promoting salicylic-dependent defense signaling response

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SUMMARY

During virus infection, the interaction of specific viral components with host factors elicits the transcriptional reprogramming of diverse cellular pathways. Alfalfa mosaic virus (AMV) establishes a compatible interaction in both tobacco and Arabidopsis hosts. Here, we show that the coat protein (CP) of AMV directly interacted with the transcription factor (TF) ILR3 of both plant species. ILR3 belongs to the basic helix–loop–helix (bHLH) family of TFs that have been proposed to participate in diverse metabolic pathways. ILR3 has previously shown to regulate NEET in Arabidopsis, a protein that plays a critical role in plant development, senescence, iron metabolism and reactive oxygen species (ROS) homeostasis. Our findings indicate that AMV CP-ILR3 interaction caused the relocation of a fraction of this TF from the nucleus to the nucleolus. The characterization of an Arabidopsis loss-of function ILR3 mutant (*ilr3.2* plants) showed that this mutant has increased ROS, PR1 mRNAs, and SA and JA contents which suggest the implication of ILR3 in the regulation of the plant defence responses. In AMV- infected wild type (wt) plants, the expression of NEET was significantly reduced whereas in *ilr3.2* mutant plants was slightly induced. In addition, SA and JA accumulation was induced in Arabidopsis wt-infected plants whereas in *ilr3.2* plants AMV infection increased JA more than ten times and SA was significantly reduced indicating an antagonist crosstalk effect. Remarkably, the accumulation levels of the viral RNAs were significantly reduced in the *ilr3.2* mutants although the virus was still able to systemically invade the plant. Our results indicate that the interactions of the AMV CP with the TF ILR3 downregulates a host factor, NEET, which in turn, would lead to activation of plant hormone responses to get an hormonal equilibrium state at which infection is maintained at a level that does not affect plant viability.

INTRODUCTION

Compatible plant-virus interactions result in systemic infections that trigger many changes in host gene expression and metabolism which can cause negative impact on normal plant development (Maule et al., 2000; Whitham et al., 2003, 2006; Pallas and Garcia, 2011; Rodrigo et al., 2012; Palukaitis et al., 2013). Gene expression changes that occur during virus infection can be elicited by the general accumulation of viral factors (Aparicio et al., 2005) but also by the interaction and/or interference of specific viral components with host factors (Culver and Padmanabhan, 2007; Mandadi and Scholthof, 2013; Garcia and Pallas, 2015). Among them, interactions between viral proteins and transcription factors (TF) can result in the transcriptional reprogramming of different cellular pathways, which makes their identification most interesting. In this way, interactions between diverse TFs of the NAC domain family and viral proteins have been described which, depending on the virus, can either enhance or inhibit virus accumulation (Olsen et al., 2005: Selth et al., 2005**;** Puranik et al., 2012; Donze et al., 2014). Remarkably, it has been recently demonstrated that a viral protein acts as a plant TF by up-regulating the regulator of cell proliferation upp-L, in turn, a TF of the basic helix-loop-helix family, to cause severe leaf malformation (Lukhovitskaya et al., 2013). Diverse studies have pointed out the implication of TFs of the WRKY family in the regulation of defence responses signalling (Kim et al., 2008; Peng et al., 2012).

Basic helix–loop–helix (bHLH) TFs comprise a family of transcriptional regulators that bind as homo- and heterodimers to specific DNA target sites, and are implicated in diverse pathways of plant metabolism and development (Heim et al., 2003; Toledo-Ortiz et al., 2003). In Arabidopsis, 147 bHLH genes have been identified and grouped into 21 families (Toledo-Ortiz et al., 2003). Arabidopsis bHLH105/ILR3 (referred to thereafter as AtILR3) belongs to the subgroup IVc (Toledo-Ortiz et al.,

2003; Heim et al., 2003), whose members are characterised by presenting a leucine zipper domain following the bHLH domain. AtILR3 is expressed in all tissues through the entire plant developmental stages. Several findings suggest that AtILR3, in combination with other regulatory proteins, might direct or indirectly participate in diverse metabolic pathways as iron and ROS homeostasis, auxin responsiveness and stress responses (Rampey et al., 2006; Long et al., 2010; Nechushtai et al., 2012). Thus, AtILR3 has been found to directly interact with Arabidopsis PYE (another bHLH TF) and BRUTUS (a putative E3 ligase protein). These two proteins are implicated in Fe metabolism by regulating the expression of genes involved in iron homeostasis (Long et al., 2010). Interestingly, a study using gain and loss-of-function Arabidopsis *ilr3* mutants (Rampey et al., 2006) indicated that this TF could regulate the expression of one gene that codes for a protein containing an iron-binding zinc finger CDGSH type domain recently identified as the plant version of NEET proteins (AtNEET) (Nechushtai et al., 2012) and three significantly homologous genes to Arabidospis *vacuolar iron transporter 1* (Kim et al., 2006), denominated *vacuolar iron transporter homologs* (VITh) (Rampey et al., 2006). NEET proteins are involved in assisting the Fe-S cluster transfer between proteins (Paddock et al., 2007) and AtNEET has been found to play a critical role in plant development, senescence, iron metabolism and reactive oxygen species (ROS) homeostasis (Nechushtai et al., 2012).

Among plant viral factors, CPs are multifunctional proteins that play major roles in most virus infection steps, including the establishment of interactions with host factors (Callaway et al., 2001; Ni and Chen-Kao, 2013; Weber and Bujarski, 2015). In this sense, the CP of alfalfa mosaic virus (AMV) is involved in regulating the replication and translation of viral RNAs, cell-to-cell and systemic movement of the virus and virion formation (Sanchez-Navarro et al., 2006; reviewed in Bol, 2005). AMV

is the only member of the genus *Alfamovirus* in the family *Bromoviridae* which, with the ilarvius genus, requires the presence of the CP in inoculum to be infectious (see Pallas et al., 2013 for a recent review). Its genome consists in three single-stranded RNAs of plus sense polarity. RNAs 1 and 2 encode the replicase subunits P1 and P2, respectively, whereas the RNA 3 encodes the movement protein (MP) and serves as a template for the synthesis of non-replicating subgenomic RNA4 (sgRNA4), which encodes the CP (Bol, 2005). In a previous work we identified a nucleolar localisation signal (NoLS) in the AMV CP and found that the cytoplasmic/nuclear-nucleolar balance of CP accumulation modulates viral expression (Herranz et al., 2012). Yet, it is still unknown whether accumulation of the CP in the nucleus/nucleolus affects general cell gene expression. Besides, a recent work identified several Arabidopsis proteins interacting with the AMV CP although the effect on the infection was only analyzed for a component of the chloroplast oxygen evolving complex of Photosystem II (PsbP) whose over-expression negatively affected to the virus accumulation (Balasubramaniam et al., 2014).

In this work we report the interaction between the AMV CP and the TF ILR3 from both Arabidopsis and *N. tabacum.* By comparing AMV infection in an Arabidopsis loss-of function ILR3 mutant (*ilr3.2* plants) with wild type (wt) plants we were able to link the activity of this TF with the hormone-based plant defence system. A model is proposed by which, upon infection, AMV CP-ILR3 interaction would downregulate a host factor, NEET, which in turn would activate ROS and SA- and JA-dependent signalling defence.

RESULTS

AMV CP interacts with ILR3 from Arabidopsis and N. tabacum.

To identify AMV CP host interacting proteins we performed yeast two hybrid (Y2H) screening using the CP as bait and an Arabidopsis leaf-specific cDNA library as a prey (Németh et al., 1998). Several clones containing almost the full-length sequence of the transcription factor *ilr3* (at3g23210) were found to grow on interaction minimal synthetic selective medium (data not shown). As we were interested in using *Nicotiana tabacum* as plant host, the NCBI database was searched for Nicotiana ILR3 homologues. We found two putative *ilr3-like* genes in *N. tabacum* that we denominated NtILR3-like1 and NtILR3-like2, respectively. NtILR3-like2 had a single-nucleotide deletion at its C-terminus causing a premature stop codon after amino acid 205, although either the bHLH as the following leucine zipper domains characteristic of subgroup IVc are conserved (Fig. S1). AtILR3 protein showed an identity of 70% with NtILR3-like1 and 57% with NtILR3-like2 (Fig. S1). To validate the original Y2H screening full length ORFs of AtILR3, a homolog from the subgroup IVc (AtbHLH115, at1g51070) and NtILR3-like1 were fused to the activation domain (pAD plasmid) and transformed into yeast cells expressing the AMV CP fused to the binding domain (pBD plasmid). After grown at 28ºC for 5 days on interaction selective medium we found that the CP specifically interacted with AtILR3 and NtILR3-like1, but not with AtbHLH115 (Fig. 1A). Empty pBD vector or expressing the tumor protein p53 (pBD:p53) were used as negative interaction controls (Fig. 1A). Besides, pBD:CP-pAD interaction was performed to rule out CP self-activation (Fig. S2). Next, we determined the subcellular localization of the TFs by transiently agro-expressing the proteins fused in frame at the C-terminus of the red fluorescent protein (dsRed). Confocal laser-scanning microscopy (CLSM) demonstrated that all three TFs were exclusively localized trough the nucleoplasma except the nucleolus (Fig. 1B, only dsRed:NtILR3-like1 and dsRed:AtbHLH115 are shown). Finally, bimolecular complementation analysis (BiFC)

was used to corroborate *in planta* CP-ILR3 interactions (Aparicio et al., 2006). Reconstituted YFP fluorescence was detected forming discrete granules exclusively in the nuclei of cells infiltrated with AMV CP and both ILR3 proteins (Fig. 2 CYFP:CP plus NYFP-AtILR3 or NYFP-NtILR3-like1) whereas no interaction was detected between AtbHLH115 and the CP (Fig. 2, CYFP:AtbHLH115 plus NYFP:CP).

It has been previously shown that the interaction between viral and host proteins can affect to their subcellular localization (e.g. Ren et al., 2005; Uhring et al., 2004; Inaba et al., 2011). To analyze whether ILR3-CP interaction during the infection could have an effect on ILR3 subcellular location, transgenic *N. tabacum* plants expressing the proteins of AMV P1 and P2 (P12 plants, Taschner et al., 1991) were inoculated with RNA transcripts from a modified AMV RNA 3 clone which expresses the GFP permitting the infected cells to be visualised (Fig. 3A, R3GFP-CPwt construct) (Sanchez-Navarro et al., 2001). At 48 hours post-inoculation (hpi) these leaves were infiltrated with *Agrobacterium* expressing AtILR3, NtILR3-like1 or AtbHLH115 fused to the dsRed. At 96 hpi leaves were examined by fluorescence microscopy to localize infection foci identified by expressing the GFP (Fig. 3B) and CLSM images were taken of nuclei from non-infected and infected cells (Figs. 3B, upper arrow and two lower arrows, respectively, and Fig 3C). In infected cells, GFP accumulates into the nucleus except the nucleolus whereas that a fraction of dsRed:AtILR3 and dsRed:NtILR3-like1 accumulated into the nucleolus, differing with their localization in non-infected cells where all three fusion protein remained in the nucleoplasm without entering into the nucleolus (Fig. 3C,only dsRed:AtILR3 is presented as representative of the three TFs.). In contrast, dsRed:AtbHLH115 located out of the nucleolus of infected cells (Fig. 3C, lower row panels). Since AMV CP accumulates into the nucleolus of infected cells (Herranz et al., 2012) we wondered whether the ILR3 fraction found into the nucleolus

would be transported to this structure as a result of its interaction with the nucleolar-traffic CP. To corroborate this hypothesis we inoculated P12 leaves with an RNA3 mutant which fails to accumulate the CP into the nucleolus as the CP lacks the NLoS (Fig. 3D R3GFP-CPNLoS) (Herranz et al., 2012) and as before, inoculated leaves were infiltrated with *Agrobacterium* expressing the TFs. Again, fluorescence microscopy was used to identify infection foci (Fig 3E) and CLSM images were taken of nuclei from infected leaves (Fig 3F). In this case, neither of the three TFs accumulated into the nucleolus of infected cells (Fig. 3F, only dsRed:AtILR3 is shown as representative image of all TFs). Finally, we established by Y2H that the CPNLoS mutant was still able to interact with NtILR3-like1 (Figure 1C). In summary our results indicate that the TFs interacting by Y2H with the CP, e.g. AtILR3 and NtILR3 but not the non-interacting AtbHLH115, were relocated towards the nucleolus of infected cells whereas this targeting failed when a mutated CP lacking the NLoS was used.

Loss of ILR3 activity activates plant defence responses.

Previous transcriptomic analysis of an Arabidopsis ILR3 loss-of-function mutant *ilr3.2* identified by Rampsey and colleagues (2006) showed altered mRNA levels of AtNEET suggesting that ILR3 might be direct or indirectly implicated in the transcription regulation of this gene. Besides, another study reported that Arabidopsis RNAi interference lines with reduced AtNEET mRNA presented enhanced accumulation of ROS suggesting that this protein is involved in ROS homeostasis (Nechushtai et al., 2012).

As previously reported, RT-PCR analysis did not detect intact ILR3 mRNA in the homozygous *ilr3.2* (Fig. 4A) (Rampsey et al., 2006). Besides, northern blot analysis showed that in our plant growth conditions, AtNEET mRNA was also slightly reduced

in *ilr3.2* mutant (Fig. 4A, NEET panel). Next, we wondered whether the reduction of NEET mRNA accumulation would be accompanied by deregulation of ROS levels. Thus, we analyzed ROS by visualizing H_2O_2 accumulation using DAB staining. DAB polymerizes in contact with H_2O_2 rendering a visible reddish-brown precipitate (Fryer et al., 2003). We found a brown precipitate in *ilr3.2* leaves correlating with the relative decrease of AtNEET mRNA (Fig. 4B). As ROS has been implicated in plant response against pathogen attack, we examined whether other defence pathways could be also being challenged in *ilr3.2* plants and found that the pathogenesis related protein 1 (PR1) mRNA, a hallmark of salicylic acid (SA) signalling activation was also clearly induced (Fig. 4A, panel PR1). Finally, quantification of the fresh weight demonstrated that in our growth conditions *ilr3.2* presented about a 30% reduction in weight than wt plants (Fig. 4C). Altogether these data showed that interfering with ILR3 function lead to activation of defence responses.

AMV infection interferes with putative activity of ILR3 and increases SA and JA biosynthesis.

An open question was whether AMV infection would induce in wt plants the same metabolic effects found in *ilr3.2* mutant. The fact that NEET mRNA expression might be regulated by ILR3, led us to identify putative NEET proteins in tobacco. NCBI databases search identified a putative protein sequence in tobacco (acc number EB680812) showing an identity of 70% with AtNEET (at5g51720) being the N-terminal part the most dissimilar domain (Fig. S1). We examined its subcellular localization by fusing the protein to the N-terminal of the green fluorescent protein (GFP) (NtNEET:GFP). Since AtNEET was previously described to accumulate in both chloroplast and mitochondria (Nechushtai et al., 2012) we co-infiltrated in *N.*

benthamiana leaves NbNEET:GFP and a mitochondrial marker (mt-rk CD3-991) (Nelson et al., 2007). CLSM showed that unlike AtNEET, the tobacco protein exclusively accumulated in chloroplasts (Fig. 5 arrowheads and arrows indicate chloroplasts and mitochondria, respectively).

To analyze the effect of AMV infection, *N. tabacum* cv Xanthi and Arabidopsis wt and *ilr3.2* plants were inoculated with AMV PV0196 isolate (DSMZ GmbH, Plant Virus Collection, Germany). In tobacco this isolate induces a reduction of the plant size accompanied by a chlorotic pattern in inoculated and upper leaves whereas that it is asymptomatic in Arabidopsis. Total RNA was extracted from inoculated and upper systemic tobacco leaves (at 2 and 4 days post-inoculation, respectively) (dpi) and from inoculated Arabidopsis leaves (at 4 dpi). In tobacco, northern blot analyses showed that NtILR3 mRNA accumulation was similar in mock and infected plants whereas that NtNEET and NtPR1 mRNAs were clearly down-regulated and up-regulated, respectively, in both inoculated and systemically-infected leaves (Fig. 6A, compare lanes M and A). The same effect was found in infected Arabidopsis wt plants (Fig. 6B, compare in wt plants lanes M and A). However, in *ilr3.2* plants AtNEET was slighty induced whereas that AtPR1 mRNA was reduced in infected respect to mock plants (Fig. 6B, compare in *ilr3.2* plants lanes M and A). On the other hand, northern blot analysis using a digoxigenin-labelled probe to detect the AMV RNA 3 corroborated that the virus accumulated in the analyzed tissues (Fig. 6A and B bottom panels). After that, we wondered if the observed reduction of NEET in infected plants would be also accompanied by deregulation of ROS. DAB staining revealed a strong brown precipitate indicative of H_2O_2 increase (Fryer et al., 2003) in both tobacco and Arabidopsis wt infected leaves that was absent in the non-infected material (Fig. 7, compare AMV and mock panels, respectively).

Considering that AMV infection lead to activation of defence responses we measured content of two hormones that modulate plant immunity; e.g. SA and Jasmonic Acid (JA). Phytohormone content was quantified in mock and inoculated leaves at 4 dpi from both Arabidopsis wt and *ilr3.2* plants. In Arabidopsis wt, SA and JA contents were increased in infected respect to healthy plants (Fig 8, wt plants, AMV and mock, respectively). The same response was observed in mock *ilr3.2* plants compared to wt plants (Fig 8, compare mock bars from wt and *ilr3.2* plants) indicating that loss of ILR3 activity correlates with induction of SA and JA biosynthesis. However, SA content was reduced whereas JA was increased more than 10 times in infected respect to mock *ilr3.2* plants (Fig 8, *ilr3.2* plants) suggesting that AMV infection can also induce JA biosynthesis independently of ILR3 activity. As found in other virus-host interactions the high JA accumulation in *ilr3.2* infected plants could antagonize the SA pathway explaining the reduction in SA and AtPR1 mRNA found in infected respect to mock *ilr3.2* plants (reviewed in Alazem and Lin, 2015; Collum and Culver, 2016).

ILR3 activity influences AMV accumulation.

Finally we analyzed whether the absence of AtILR3 had effect on AMV infection. Arabidopsis wt and *ilr3.2* plants were inoculated with AMV PV0196 isolate virion particles and northern blot analysis using a digoxigenin-labelled AMV CP (Fig. 9A) ORF plus quantification of the blot signals using the Image J software were carried out to measure virus accumulation. Fig. 9A shows the northen blot of upper non-inoculated leaves while the graph in Fig. 9B illustrates that AMV RNAs accumulation was reduced 40% in *ilr3.2* respect to wt inoculated leaves at 4 dpi and around 30% in upper non-inoculated leaves at 10 dpi. This experiment was repeated three times with similar results. Overall, our results indicate that loss of AtILR3 function activates plant

defence signalling response in Arabidopsis although the virus escapes the defence response being still able to move systemically infecting the whole plant at a more reduced accumulation level.

DISCUSSION

Plant viruses usurp a large number of host factors/resources in their own benefit for their survival whereas the infected plant activates a whole series of responses to protect from the negative impact caused by the pathogen (Culver and Padmanabhan, 2007; Mandadi and Scholthof, 2013; Garcia and Pallas, 2015). In this work we demonstrated that the interaction of a viral coat protein with a transcription factor might participate in activating the plant defence response in the infected plants although this host response does not prevent the virus invading the plant.

The CP of AMV is a multifunctional protein that plays essential roles in the viral cycle: from regulating the replication and translation of viral RNAs to cell-to-cell and systemic movement and encapsidation (reviewed in Bol, 2005 and Pallas et al., 2013). In the present work we report the interaction between the CP and ILR3, a TF belonging to the bHLH family. This interaction leads to the relocation of part of the ILR3 protein pool into the nucleolus and interestingly, this interaction was observed with ILR3 from two plant species suggesting that this phenomenon can be a general feature of AMV infection. We have previously shown that the AMV CP accumulates at the cytoplasm and in the nucleus/nucleolus of infected cells (Herranz et al., 2012) and here we present evidence that the ILR3-nucleolar translocation is probably driven by viral protein transport towards the nucleolus. Subcellular localization changes of host factors induced by interactions with viral proteins have been previously reported. For instance, in Arabidopsis infected with cucumber mosaic virus the cytoplasmic catalase 3 is

relocalizated into the nucleus through its interaction with the 2b protein (Inaba et al., 2011) whereas that the p19 protein of tomato bushy stunt virus interacts with three proteins of the AYL family causing their transport from the nucleus to the cytoplasm (Uhring et al., 2004). On the other hand, the CP of Turnip crinkle virus interacts with the TF TIP preventing its nuclear localization (Ren et al., 2004).

One relevant question is raised, that of how the CP-ILR3 interaction affects the TF function. Although it has been established that bHLH TFs function as homo- and heterodimers (Heim et al., 2003; Toledo-Ortiz et al., 2003), and several findings have led to the hypothesis that ILR3 might function in combination with other bHLH TFs (Long et al., 2010), no direct targets of ILR3 have yet been identified. Experiments conducted to determine the AtILR3 function with gain- and loss-of-function Arabidopsis mutants have reported that the mRNA levels of AtNEET were altered in both mutant types suggesting that this TF might directly or indirectly influence NEET expression (Rampsey et al., 2006). Our northern analysis showed that the mRNA accumulation of NEET in both tobacco and Arabidopsis was slightly down-regulated during the course of infection resembling the Arabidopsis loss-of-function *ilr3-2* (Rampsey et al., 2006). However, taking in account the critical role of NEET in Arabidopsis metabolism we cannot rule out the possibility that others TFs might function redundantly to regulate NEET mRNA transcription.

AtNEET has previously been localized in both mitochondria and chloroplast and has been found to be a key regulator in plant development, senescence, Fe metabolism and ROS homeostasis (Nechushtai et al., 2012). However, contradictory results have been reported about its subcellular localization since a recent study concluded that AtNEET is translocated into choloroplast but not into mitochondria (Su et al., 2013). In

the case of NtNEET, we found that the protein was localized only in chloroplasts. Transport of proteins to mitochondria and chloroplasts is achieved through N-terminal transit peptides and accordingly, AtNEET and NtNEET contain the proposed N-terminal chloroplast transit peptide cleavage motif V-R/K-A-E (Su et al., 2013)- However, this N-terminal region presents most of the differences observed in their sequences. The absence of NtNEET in the mitochondria could be explained assuming that the N-terminal region of NtNEET lacks the mitochondrial transit peptide.

Our results link chloroplast function with viral infection. The chloroplast is a key organelle to generate plant defence signalling molecules including ROS and SA (Torres, 2010; Spoel and Dong, 2012). In fact, several viruses encode proteins interacting with components of the photosynthetic machinery and in some cases it has been found that defense host response activation is prevented trough these interactions (Abbink et al. 2002; Jimenez et al. 2006; Bath et al., 2013). In this sense, it has been recently reported that AMV CP through its interaction with the chloroplast-targeted PsbP also might favor AMV replication by sequestering the host protein in the cytosol which would impede the activation of plant defense responses (Balasubramaniam et al., 2014).

ROS, SA and JA concentration are increased in healthy *ilr3.2* plants indicating that ILR3 activity would participate in plant defence response modulation (Fig. 10). Our results suggest that, through the CP-ILR3 interaction, AMV might interfere with the TF's function causing downregulation of NEET expression. This in turn, would lead to activation of ROS and SA defence responses. Further, activation of JA biosynthesis would be partially independent of ILR3 function (Fig. 10). Increasing of ROS and activation of SA signalling pathway has been found in diverse compatible virus-host interactions (Whitham et al., 2006). In fact, in compatible host-virus interactions, the expression of the majority of defense-related genes is induced by a SA-dependent

signalling pathway (Huang et al., 2005). On the other hand, the high JA concentration reached in *ilr3.2* infected plants might antagonize SA pathway leading to the strong downregulation of AtPR1 mRNA observed in these plants. Different studies have reported that depending upon the concentration of each hormone, SA and JA pathways can have antagonistic or synergic effects (reviewed in Pieterse et al., 2012; Collum and Culver, 2016). In this sense, it has been found that in some compatible virus-host interactions early components of the SA pathway may be regulated by JA (reviewed in Alazem and Lin, 2015).

In summary, our results show that AMV infection activates plant defence pathways which would negatively affect virus accumulation without impeding the systemic infection of the host. A model is proposed (Fig. 10) by which, upon infection, AMV CP-ILR3 interaction would modify the subcellular location of the TF downregulating a host factor, NEET, that in turn would activate ROS and SA- and JA-dependent signalling defence, being the latter alternatively independent of ILR3.

EXPERIMENTAL PROCEDURES

Yeast two hybrid screening

Arabidopsis cDNA library fused to the GAL4 activation domain (AD) into pGADT7 has been previously described (Németh et al., 1998). Plasmids pBD:CP and pBD:CPNLoS containing the full-length AMV CP variants fused to the GAL4 binding domain (BD) into pGBKT7 plasmid have been described (Herranz et al., 2012). Yeast two-hybrid (Y2H) screening was performed with the Matchmaker Gal4 Two-hybrid System 3 (Clontech) following manufacturer´s recommendations. Briefly, yeast reporter strain AH109 (Clontech) was sequentially transformed with pBD:CP and pGAD:cDNA

and co-transformants were selected by culturing on minimal synthetic medium lacking leucine and tryptophan (-LW). Positive interactions were selected by culturing on medium lacking leucine, tryptophan, adenine and histidine (-LWHA). Cultures were kept at 28ºC for 5 days. pAD:cDNA clones from positive interactions were rescued and subjected to DNA sequencing. Full length ORF of AtILR3, AtbHLH115 and NtILR3-like1 were PCR amplified with specific primers (Table S1), cloned into pGADT7 plasmid and transformed in AH109 carrying the empty pGBKT7 (pBD), pBD:CP or pBD:p53 plasmids. Reconfirmation of the interactions was carried out by growing cells in (-LWHA) medium for 5 days at 28ºC.

BiFC and subcellular localization analysis

AtILR3, AtbHLH115, NtILR-like1, and NtNEET ORFs were amplified with specific primers (Table S1) designed for cloning using the Gateway System (Invitrogen). Amplified products were recombined into a donor vector using the BP reaction and then transferred into binary destination vectors expressing the full-length Red fluorescent protein (dsRed) or the green fluorescent proteins (GFP) for subcellular localization studies and the N-terminal and C-terminal part of the yellow fluorescent protein (YFP) for BiFC analysis, following manufacturer´s recommendations. The fusion proteins generates were as follows: dsRed:AtILR3, dsRed:AtbHLH115, dsRed:NtILR3-like1, NtNEET:GFP, NYFP:AtILR3 NYFP:NtILR3 and CYFP:AtbHLH115. Plasmids expressing the AMV CP fused to the N-terminal and C-terminal parts of the YFP (NYFP:CP and CYFP:CP) and the mitochondrial marker mt-rk CD3-991 have been previously described (Aparicio et al., 2006; Nelson et al., 2007). All binary vectors were transformed into *Agrobacterium tumefaciens* C58 cells. Cultures were diluted at 0.2 OD_{600} in infiltration solution (10 mM MES pH 5.5, 10 mM

$MgCl_2$) and infiltrated into 3 week old *N. benthamiana* plants. For nuclei staining 10 mg/mL 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) was infiltrated into *N. benthamiana* leaves 1 h before observation.

Confocal images were taken at 48 hours after agro-infiltration with a microscope Zeiss LSM 780 AxiObserver. All images correspond to single slices of 1.8 μm thickness of epidermal cells. Excitation and emission wavelengths were 359 and 457 nm for DAPI, 488 and 508 nm for GFP, 514 and 527 nm for YFP, 545 and 572 nm for dsRed and 488 and 750 nm for chloroplast visualization, respectively.

Plant growth conditions and virus inoculation

Arabidopsis ecotype Col-0 wt and *il3.2*, *N. tabacum* cv Xanthi, *N. benthamiana* and *N. tabacum* cv Samsun P12 plants (Taschner et al., 1991) plants were grown in 12 cm pots diameter in a growth chamber at 24ºC with a photoperiod of 24ºC-16 h light/20ºC-8 h dark.

To analyze AMV infection effects on Arabidopsis or *N. tabacum* cv Xanthi, two leaves of 3 weeks old plants were mechanically inoculated with purified virions (1 mg/ml) of AMV PV0196 isolate (DSMZ GmbH, Plant Virus Collection, Germany) in 30 mM sodium phosphate buffer pH 7 or with buffer alone (mock plants).

Plasmids px032/GFP-MP-CP and px032/GFP-MP-CP(K5-13:A) (Sánchez-Navarro et al., 2001; Herranz et al., 2012) (here labelled R3CPwt-GFP and R3CPNLoS-GFP, respectively) were used to perform ILR3 re-localization studies in P12 plants. For inoculation purposes, *Pst*I-linearized plasmids were transcribed with T7 RNA polymerase (Takara) following manufacturer's recommendations. Leaves were mechanically inoculated with 1 ug/leaf of the corresponding transcripts and *A.tumefaciens* C58 cultures expressing the corresponding fusion proteins were prepared

in infiltration buffer at 0.1 OD_{600} and infiltrated at 48 hours post-inoculation (dpi). Images of infection foci were taken at 4 dpi with a microscope Zeiss LSM 780 AxiObserver.

Cloning of host genes for northern blot analysis

NCBI and http://sydney.edu.au/science/molecular_bioscience/sites/benthamiana/ database were searched for tobacco NEET and PR1, genes. Multiple Sequence Alignment by *CLUSTALW* program (Thompson et al., 1994) was used to analyze homologies between Arabidopsis and tobacco genes. RT-PCR reactions were carried out from RNA extractions with specific primers (Table S1). Amplified products were cloned into pTZ57R/T plasmid (ThermoFisher Scientific). After digestion with *Xba* I restriction enzyme (ThermoFisher Scientific), linearized plasmids were used as templates to transcribe digoxigenin-labelled probes. Transcriptions were carried out with T7 RNA polymerase (Takara) following manufacturer´s recommendations.

Detection of host mRNAs and viral RNAs was carried out by northern blot analysis. In the case of *N. tabacum* inoculated leaves were harvested at 2 dpi whereas that upper systemic leaves were collected at 4 dpi. In the case of Arabidopsis inoculated leaves were harvested at 4 dpi and upper systemic leaves were at 10 dpi. Leaves were grounded in liquid nitrogen with mortar and pestle and total RNA was extracted from 0.1 g leaf material using Trizol Reagent (Sigma). 10 ug and 1 ug of total RNA, for mRNA and viral RNA detection respectively, were denatured by formaldehyde treatment and analyzed by northern blot hybridization as described previously (Sambrook *et al.,* 1989). Viral RNAs were visualized on blots using a digoxigenin-labelled riboprobe corresponding to the AMV CP gene. Synthesis of the digoxigenin-labelled riboprobe, hybridization and digoxigenin detection procedures were carried out as previously described (Pallas et al., 1998).

DAB staining

DAB staining was carried out as previously described (Liu et al., 2012) with some modifications. Samples were analyzed at the same time than northern blot studies. To prepare DAB solution, 3,3-diaminobenzidine (Sigma) was diluted at 1 mg/ml in H_2O at pH 3.6 by vigorous shaking at 37ºC for at least 30 min. Leaves were infiltrated with DAB solution with a syringe and placed in Petri dish containing paper towels saturated with a fixative solution (ethanol/acetic acid/glycerol 3:1:1 V/V/V) for 3-4 days at room temperature until green colour disappeared. Bright field images were recorded with a Nikon Eclipse E600.

Hormone analysis

For SA and JA quantification, mock and AMV inoculated leaves from Arabidopsis wt and *ilr3.2* plants were collected at 4 dpi. Each sample containing a mixture of leaves from three plants was ground in liquid nitrogen with mortar and pestle. Material (100 mg fresh weight) was suspended in 80% methanol-1% acetic acid containing internal standards and mixed by shaking during one hour at 4ºC. The extract was kept a -20ºC overnight and then centrifuged and the supernatant dried in a vacuum evaporator. The dry residue was dissolved in 1% acetic acid and passed through an Oasis HLB (reverse phase) column as described in Seo et al (2011).

The dried eluate was dissolved in 5% acetonitrile-1% acetic acid, and the hormones were separated using an autosampler and reverse phase UHPLC chromatography (2.6 µm Accucore RP-MS column, 50 mm length x 2.1 mm i.d.; ThermoFisher Scientific) with a 5 to 50% acetonitrile gradient containing 0.05% acetic acid, at 400 µL/min over 14 min. - For SA and JA quantification, the dried eluate was dissolved in 5% acetonitrile-1% acetic acid, and the hormones were separated using an autosampler and reverse phase UHPLC chromatography (2.6 µm Accucore RP-MS

column, 50 mm length x 2.1 mm i.d.; ThermoFisher Scientific) with a 5 to 50% acetonitrile gradient containing 0.05% acetic acid, at 400 µL/min over 14 min

The internal standard for SA quantification was the deuterium-labeled hormone, whereas for JA, the compound dhJA was used. The hormones were analyzed with a Q-Exactive mass spectrometer (Orbitrap detector; ThermoFisher Scientific) by targeted Selected Ion Monitoring (SIM). The concentrations of hormones in the extracts were determined using embedded calibration curves and the Xcalibur 2.2 SP1 build 48 and TraceFinder programs. Measurements were done by triplicate.

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SUPPORTING INFORMATION

Table S1. List of primers and plasmids used in this study.

Fig. S1. Amino acid sequence alignments of ILR3 and NEET proteins of Arabidopsis and *N. tabacum*.

Fig. S2. Analisis of putative Y2H self-activation by AMV CP. AH109 yeast cells co-transformed with the indicated plasmids were spotted on minimal medium lacking leucine and tryptophan (-LW) or leucine, tryptophan, histidine and adenine (-LWHA). Colony growth was only detected with the pair pBD:CP and pAD:CP confirming that AMV CP does not self-activate yeast growth.

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FIGURE LEGENDS

Fig. 1. Arabidopsis and tobacco ILR3 interacts with AMV CP. (A) AMV CP and (C) lacking the NLoS interaction with AtILR3, AtbHLH115 and NtILR3-like1 transcription factors by yeast two hybrid system. AH109 yeast cells co-transformed with the indicated plasmids were spotted on minimal medium lacking leucine and tryptophan (-LW) or leucine, tryptophan, histidine and adenine (-LWHA) to confirm proper co-transformation and positive interactions, respectively. (B) Nuclei from cells expressing NtILR3-like1 and AtbHLH115 fused to dsRed and stained with DAPI are shown by CLSM in the red (RED) and blue (DAPI) channels. Overlay images confirmed that the TFs localized exclusively in the nucleoplasm.

Fig. 2. BiFC analysis of CP-ILR3 interaction. CLSM images of nuclei from epidermal cells co-infiltrated with the constructions indicated on the left and stained

with DAPI are shown in the yellow (YFP) and blue (DAPI) channels. Overlay panels are the superposition of YFP and DAPI over the bright field images. YFP reconstitution was exclusively found in nuclei (arrows). BiFC nnomenclature of the plasmids is as follows: NYFP and CYFP refer to the N-terminal and C-terminal fragments of the YFP and in all constructs the YFP tag is attached to the N-terminus of the protein. Bars = 10 µm.

Fig. 3. **AMV infection promotes nucleolar relocalization of AtILR3**. (A and D) Schematic representation showing the modified AMV RNA 3 with the wt CP (R3GFP-CPwt) or the mutated CP lacking the NLoS (R3GFP-CPNLoS and expressing the GFP. (B and E) Images of one infection focus denoted by the accumulation of GFP. (C) CLSM images in the green (GREEN), red (RED) and blue (DAPI) channels of nuclei from non-infected and infected cells with R3GFP-CPwt and transiently expressing the TFs indicated in the right of each rows of panels. White arrows indicate the nucleolus. (F) CLSM images of a nucleus of a cell infected with R3GFP-CPNLoS and transiently expressing dsRed:AtILR3. In this case, the TF does not accumulate in the nucleolus (white arrow). In all images bar: 5 um.

Fig. 4. **Characterization of Arabidopsis *ilr3.2* mutant plants.** (A) Northern blots to detect mRNA accumulation of several genes (indicated on the left) in wt and *ilr3.2* plants. Ethidium bromide (EtBr) staining of ribosomal RNAs was used as RNA loading control. (B) DBA staining of leaves from wt and *ilr3.2* plants to reveal H_20_2 accumulation (dark brown precipitate). (C) Plant weight of wt and *ilr3.2* plants. Rossetes from 21 days old plants were cut from roots and weighed. Asterisk indicate significant difference from the wt (*$P < 0.05$) using the Paired t-test (n=25). Error bars represent standard error of the mean.

Fig. 5. Subcellular localization of NtNEET protein. Magnified image of a cell co-expressing NtNEET:GFP (GREEN CHANNEL) and the mitochondrial marker mt-rk CD3-991 (RED CHANNEL). Auto fluorescence of chlorophyll in chloroplast is shown in magenta (CHLOROPHYLL). Overlay image reveal that NtNEET accumulates in chloroplast (white arrowhead) but not in mitochondria (arrows).

Fig. 6. AMV infection activates plant defence responses. Northern blots to detect mRNA accumulation of several genes (indicated on the middle of panels) from mock and AMV inoculated plants (M and A, respectively) in *N. tabacum* (A) and Arabidopsis (B). Inoculated and upper systemic leaves in tobacco and inoculated leaves in Arabidopsis wt and *ilr3.2* plants were analyzed (indicated on the top). Ethidium bromide (EtBr) staining of ribosomal RNAs was used as RNA loading control. Northern blot to detect the AMV RNA 3 (lower panel) was used to corroborate viral accumulation.

Fig. 7. DBA staining of *N. tabacum* and Arabidopsis wt plants. Inoculated or upper systemic leaves (indicated on the left) from healthy (mock) and infected (AMV) plants were infiltrated with DAB solution to visualize H_2O_2 accumulation.

Fig. 8. SA and JA content in Arabidopsis wt and *ilr3.2* plants. Hormone content was measured in mock and AMV inoculated leaves at 4 dpi. Bars represent mean and standard deviation of values obtained from three biological replicates.

Fig. 9. **ILR3 activity affects to AMV accumulation in Arabidopsis.** (A) Northern blot corresponding to five Arabidopsis wt and *ilr3.2* plants to detect viral RNA 3 and 4 (R3 and R4). Ethidium bromide (EtBr) staining of ribosomal RNAs was used as RNA loading control. (B) Graphic showing the average of viral RNAs accumulation measured of inoculated and upper systemic leaves (inoc and upper, respectively) in wt and *ilr3.2* plants. (*$P<0.05$ using the Paired t-test, n=5).

Fig. 10. Hypothetical model to illustrate the effect of CP-ILR3 interaction on plant defence response. In healthy plants ILR3 activity may regulate NEET levels contributing to the repression of ROS and SA signalling pathways. Additionally, ILR3 would participate regulating JA biosynthesis. In AMV infection, interaction between the CP and ILR3 might reduce ILR3 activity leading to repression of NEET mRNA accumulation and activation ROS and SA signalling. At the same time AMV infection would induce JA biosynthesis independently of ILR3 activity.