IARA PINHEIRO CALIL

CHARACTERIZATION OF TRANSCRIPTIONALLY ACTIVE ATWWP1 NUCLEAR BODIES THAT CONFER PARTIAL IMMUNITY AGAINST BEGOMOVIRUSES

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS – BRASIL
2017

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
C153c
2017

Calil, Iara Pinheiro, 1987-
Characterization of transcriptionally active AtWWP1 nuclear bodies that confer partial immunity against begomoviruses / Iara Pinheiro Calil. – Viçosa, MG, 2017.
viii, 152f. : il. ; 29 cm.

Inclui anexos.
Orientador: Elizabeth Pacheco Batista Fontes.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Begomovírus. 2. DNA - Análise. 3. Proteínas. 4. Imunidade. I. Universidade Federal de Viçosa. Departamento de Bioquímica e Biologia Molecular. Programa de Pós-graduação em Genética e Melhoramento. II. Título.

CDD 22 ed. 632.8

IARA PINHEIRO CALIL

CHARACTERIZATION OF TRANSCRIPTIONALLY ACTIVE ATWWP1 NUCLEAR BODIES THAT CONFER PARTIAL IMMUNITY AGAINST BEGOMOVIRUSES

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de *Doctor Scientiae*.

APROVADA: 06 de fevereiro de 2017.

Francisco Murilo Zerbini Junior	João Paulo Batista Machado
Welison Andrade Pereira	Sergio Herminio Brommonschenkel (Coorientador)

Elizabeth Pacheco Batista Fontes
(Orientadora)

Aos meus amados pais Cézar e Cássia

DEDICO

AGRADECIMENTOS

Agradeço à Universidade Federal de Viçosa, pelo suporte técnico e científico a meu aprendizado e contribuição para minha formação.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelo apoio financeiro.

À professora Elizabeth Fontes, pela oportunidade de estagiar em seu laboratório desde a iniciação científica, pela orientação, paciência e pelo exemplo de profissionalismo.

À Joanne Chory, pela oportunidade de desenvolver parte deste projeto em seu laboratório.

Aos colegas do LBMP e do PBIO/SALK pela amizade, pela excelente convivência e pela oportunidade de aprendizado durante as inúmeras discussões científicas.

Aos meus familiares e amigos, pelo carinho e por dividirem comigo cada momento de alegria e de dificuldade.

Agradeço sobremaneira a Deus porque d'Ele, por Ele e para Ele são todas as coisas.

ÍNDICE

ABSTRACT

CALIL, Iara Pinheiro, D.Sc., Universidade Federal de Viçosa, February 2017. **Characterization of transcriptionally active atwwp1 nuclear bodies that confer partial immunity against begomoviruses**. Adviser: Elizabeth Pacheco Batista Fontes. Co-Advisers: Anésia Aparecida dos Santos and Sergio Herminio Brommonschenkel

As DNA viruses, which replicate in the nucleus of infected cells, the bipartite begomoviruses (*Geminiviridae* family) encode the nuclear shuttle protein to facilitate the translocation of viral DNA from the nucleus to the cytoplasm via nuclear pores. This intracellular trafficking of NSP-DNA complexes is accessorized by the NSP-interacting GTPase (NIG) at the cytosolic side. Here, we report the characterization of AtWWP1, a WW domain-containing protein, identified as a NIG partner. In the chapter II, we demonstrated that AtWWP1 forms nuclear bodies (NBs) via its WW domains and relocates NIG from the cytoplasm to the nucleus where it is confined to AtWWP1-NBs.Therefore, the NIG-AtWWP1 interaction, which also occurs via the WW domains, may interfere with the NIG pro-viral function that is associated with its cytosolic localization. Consistent with this hypothesis, loss of *AtWWP1* function debilitates further the plant upon begomovirus infection and overexpression of *AtWWP1* confers tolerance to begomovirus. The antiviral function of AtWWP1-NBs, however, may be antagonized by viral infection, which was demonstrated to induce either a decrease in the number or disruption of AtWWP1-NBs. Our data established that AtWWP1 organizes nuclear structures as nuclear foci, which provide intrinsic immunity against begomovirus infection. Nevertheless, the underlying biochemical function of these AtWWP1-NBs has yet to be elucidated. In Chapter III, we demonstrated that AtWWP1-NB co-localizes with CDKC;2-NB, which has been shown to be involved in transcription and RNA processing. Like CDKC2, which modulates the phosphorylation status of RNA polymerase II C-terminal domain (CTD-RNA pol II), we showed that AtWWP1 interacts with CTD-RNA pol II within NBs. AtWWP1-NBs were disintegrated into diffuse pattern or converted to larger structures upon treatment with transcriptional inhibitors, a feature that resembles the CDKC2-NB dynamic organization, which depends on the transcriptional status of the cells. As further evidence for a role in transcription, we also demonstrated that

AtWWP1 displays DNA binding activity and AtWWP1-NBs associate with active chromatin regions. Accordingly, the manipulation of the *AtWWP1* levels promoted an enrichment of differentially expressed transcriptional factors in transgenic lines. Collectively, our data establish that the nuclear bodies formed by AtWWP1 are associated with active gene expression or co-transcriptional RNA processing.

RESUMO

CALIL, Iara Pinheiro, D.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Caracterização de corpos nucleares transcricionalmente ativos, formados pela proteína AtWWP1, os quais conferem imunidade parcial contra Begomovirus.** Orientadora: Elizabeth Pacheco Batista Fontes. Coorientadores: Anésia Aparecida dos Santos e Sergio Herminio Brommonschenkel

Begomovírus bipartidos (Familía *Geminiviridae*) - grupo de vírus de DNA que se replica no núcleo de células infectadas – codifcam a proteína de movimento NSP (*nuclear shuttle protein*) que facilita a translocação do DNA viral do núcleo para o citoplasma, através dos poros nucleares. O tráfego intracelular do complexo NSP-DNA é assessorado pela proteína NIG (*NSP-interacting GTPase*), localizada no compartimento citoplasmático celular. Nesta investigação, é descrita a caracterização de AtWWP1, uma proteína dotada de dois domínios WW, identificada por sua interação com NIG. No capítulo II foi demonstrado que AtWWP1 forma corpos nucleares (NBs) por meio de seu domínio WW e é capaz de relocar NIG do citoplasma para o núcleo, confinando-a em corpos nucleares. Portanto, a interação AtWWP1-NIG, mediada pelo domínio WW de AtWWP1, pode interferir com o papel pro-viral de NIG durante a infecção o qual é associado à sua localização citoplasmática. Consistente com essa hipótese, a perda de função de *AtWWP1* em Arabidopsis resulta em aumento de suscetibilidade em resposta à infecção por begomovírus, ao passo que a superexpressão de *AtWWP1* confere tolerância à infecção viral. Entretanto, a função antiviral de AtWWP1-NB pode ser antagonizada durante a infecção viral, uma vez que foi observado um decréscimo ou desorganização dos corpos nucleares de AtWWP1 em células infectadas. Os dados apresentados no capítulo II demonstraram que AtWWP1 se organiza em estruturas subnucleares as quais conferem imunidade intrínseca contra infecção por begomovírus. Contudo, a função molecular dos corpos nucleares de AtWWP1 não foi elucidada. No capítulo III, foi demonstrado que AtWWP1 co-localiza em corpos nucleares com a proteína CDKC2, cuja função celular é associada à transcrição e processamento de RNA. CDKC2 modula o estado de fosforilação do domínio C-terminal da RNA polimerase II (CTD-RNAPII). Foi demonstrado que AtWWP1 também interage com CTD-RNAPII em NBs. Os corpos nucleares de AtWWP1 foram desfeitos ou reorganizados em corpos nucleares maiores após tratamentos

com inibidores de transcrição e fosforilação; tal comportamento se assemelha ao observado em CDKC2-NBs, cuja organização dinâmica dos corpos nucleares depende do status transcricional da célula. Como evidência adicional de seu papel na transcrição celular, foi demonstrado que AtWWP1 possui capacidade de ligação a DNA e que seus NBs estão associados à região de eucromatina. Consistente com os resultados previamente obtidos, a alteração nos níveis transcricionais de *AtWWP1* em linhagens transgênicas resultou no enriquecimento de fatores de transcrição diferencialmente expressos. Coletivamente, os dados obtidos neste trabalho demonstram que AtWWP1 forma corpos nucleares correspondentes a sítios ativos de expressão gênica ou de processamento de RNA acoplado à transcrição.

GENERAL INTRODUCTION

Plants, as sessile organisms, are constantly exposed to biotic and abiotic stresses that affect their growth and development. As a result, plant survival depends on the ability to recognize and efficiently respond to these threats. Among the biotic factors, viruses cause many economically important diseases in all parts of the world, limit productivity of relevant crops and deteriorate crop quality, representing a serious threat to global food security (Machado *et al*., 2017).

Plants employ multiple defense mechanisms to restrict viral replication and movement, such as gene silencing, immune receptor signaling, hormone-mediated defense, protein degradation and regulation of metabolism (Incarbone and Dunoyer, 2013). In virus–plant interactions, one of the major mechanisms for plant antiviral immunity relies on RNA silencing, which is often suppressed by co-evolving viral suppressors, thus enhancing viral pathogenicity in susceptible hosts. In addition, plants use nucleotide-binding leucine-rich repeat (NB-LRR) domain-containing resistance proteins, which recognize viral effectors and activate effector-triggered immunity (ETI) in a defense mechanism similar to that employed in non-viral infections (Mandadi and Scholthof, 2013). Plants also use innate pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) to limit viral infection (Kørner *et al*., 2013). More recently, a transmembrane immune receptor, which is structurally similar to co-receptor-like kinases involved in PTI, has been shown to activate host translation suppression to fight DNA viruses, a newly discovered mechanism for antiviral defenses in plants (Zorzatto *et al*., 2015). Viral infections can also lead to hormonal disruption, which manifests as simultaneous induction of many antagonistic hormones and triggering of defense responses (Alazem and Lin, 2015).

Virus–host interactions can aberrantly regulate phytohormone pathways, leading to disease development and hormone-mediated defensive responses. Plants employ the ubiquitin–proteasome pathway (UPS) as an antiviral defense strategy and, concomitantly, viruses have been reported to exploit the UPS to induce, inhibit or modify ubiquitin (Ub)-related host proteins (Alcaide-Loridan and Jupin, 2012).

Geminiviruses (*Geminiviridae* family) are among the most devastating group of plat pathogens worldwide, being one of the largest and most relevant families of plant viruses (Scholthof *et al*., 2011; Hanley-Bowdoin *et al.,* 2013). They present circular single-stranded DNA and replicate in the nuclei of infected plant cells. Due to their limited coding capacities (four to eight genes), the geminiviruses rely on virus-host intermolecular interactions, which are required for gene expression, replication and movement. Accordingly, geminiviruses are reported to manipulate and interfere in a wide range of host cellular pathways including cell cycle, ubiquitination and ubiquitination-like pathways, hormone signaling and RNA silencing-based plant defense to achieve a successful infection (Hanley-Bowdoin *et al*. 2013, Calil and Fontes, 2016). The identification and characterization of host proteins involved in geminivirus infection pathways, together with knowledge in plant virology will allow the development of antiviral defense strategies aiming crop protection.

Begomovirus, the largest and most economically relevant genera of the *Geminiviridae* family, encompasses viruses with either monopartite or bipartite genomes that are transmitted by whiteflies (*Bemisia tabaci*). The two genomic components of bipartite begomoviruses are designated DNA-A and DNA-B. DNA A typically encodes four/five proteins: Rep and REn involved in replication; AC4, encoding a suppressor of silencing; TrAP involved in transcriptional activation and silencing suppression and CP, the coat protein. A sixth ORF AV2 is found in the Old

World bipartite begomoviruses, but not in the New World viruses. DNA-B encodes two movement proteins, the nuclear shuttle protein, NSP (BV1), and the movement protein, MP (BC1), both required for systemic infection (Rojas *et al*., 2005; Fondong, 2013).

Prior to cell-to-cell movement, geminiviruses must enter into the plant cell nucleus to replicate its genome as well as to make use of the host transcriptional machinery. Consequently, they require movement proteins to move the newly replicated DNA from the nucleus to the cytosol via nuclear pores and then to adjacent cells via plasmodesma. Compelling evidence indicate that NSP binds to newly-synthesized viral DNA and facilitates its movement across the nuclear envelope, whereas MP moves the viral DNA to adjacent cells, mediating cell-to-cell transportation via plasmodesmata as well as long-distance transfer via phloem (Lazarowitz and Beachy, 1999; Rojas *et al*., 2005). Additionally, NSP acts as a suppressor of the immune receptor NSP-interaction kinase 1 (NIK1) interfering with its kinase activity (Fontes *et al*., 2004; Gouveia *et al*., 2016; Machado *et al*., 2017).

In order to promote nucleocytoplasmic trafficking of viral DNA, NSP may interact with a diverse range of host factors, including components of nuclear transport machinery and nuclear pore complex (NPC).One of host NSP partners, designated NIG (NSP-interacting GTPase), may function as cofactor for NSP function, facilitating the intracellular transport of viral DNA-N SP complexes from to the nuclear envelope to the cytoplasm where it is replaced by MP (Carvalho *et al*., 2008a; 2008b). Evidence for NIG function in the viral DNA-NSP trafficking relies on the demonstration that NIG interacts *in vivo* and *in vitro* with NSP, is capable of moving NSP-DNA complexes from the nucleus to the cytoplasm and functions as a pro-viral factor during begomovirus infection (Carvalho *et al*., 2008a; 2008b).

Successful infection by a plant virus depends on spreading their genomes between cells and throughout the organism. Thus, the identification of host factors involved in virus movement, direct or indirectly targeted by virus-encoded movement proteins, is crucial for the development of novel antiviral strategies. Additionally, dissecting the molecular network of virus-host interactions will help to understand basic cellular processes in plant biology, including replication, gene expression, intra- and intercellular transport of macromolecules and plant antiviral defenses.

Due to the inter and intracellular transport function, NSP from begomoviruses represents an excellent probe for identifying components of the basic cell machinery as this viral protein may interact with host factors in different compartments. Accordingly, NSP has been shown to interact with an *Arabidopsis thaliana* nuclear acetylase, designated nuclear shuttle protein interactor (AtNSI; Carvalho and Lazarowitz, 2004), the nuclear Histone H3 (Zhou, Y. *et al.,* 2011), plasma membrane receptor kinases, designated NIKs (Mariano *et al*., 2004; Fontes *et al*., 2004) and the cytosolic GTPase NIG (Carvalho *et al*., 2008a). Although some of these NSP-interacting factors have been extensively characterized (Hanley-Bowdoin *et al*., 2013; Machado *et al*., 2015), the nucleocytoplasmic transport function of NIG is poorly understood and the characterization of the potential NSP-interacting immune hub is far from of being complete. The goal of the present investigation was two-fold: (i) to get insights into the NIG function by investigating the function of potential NIG partners and (ii) to exploit potential interactions mediated by NIG partners into the NSP-based network of protein-protein interactions. In the chapter II, an NIG-interacting WW domain-containing protein, named AtWWP1, was identified. AtWWP1 was demonstrated to sequester NIG in nuclear bodies, impairing the cytosolic pro-viral function of NIG, thereby enhancing tolerance against begomoviruses. In chapter

III, the AtWWP1 biological role was further investigated and AtWWP1 was implicated as a transcriptionally active nuclear body-forming protein, which may support additional immune functions. Additionally, crucial interactions between AtWWP1 and host factors were identified and placed into the context of the NSP-interacting protein network. The chapter I, published in Annals of Botany (Calil and Fontes, 2016), describes the state-of-art of the antiviral immunity mechanisms in plants. The supplementary material includes two antiviral immunity-related reviews, in which the author of this thesis shares the first authorship (Machado *et al*., 2017; Gouveia *et al.*, 2016).

References

Alazem M, Lin NS. (2015) Roles of plant hormones in the regulation of host-virus interactions. *Mol. Plant Pathol.* 16, 529-540.

Alcaide-Loridan, C, Jupin, I. (2012) Ubiquitin and plant viruses, let's play together! *Plant Physiol*. 160, 72–82.

Calil, I.P., Fontes, E.P.B. (2016) Plant immunity against viruses: antiviral immune receptors in focus. *Ann Bot*. 118, 200-213.

Carvalho, C.M., Fontenelle, M.R., Florentino, L.H., Santos, A.A., Zerbini, F.M., Fontes, E.P.B. (2008a) A novel nucleocytoplasmic traffic GTPase identified as a functional target of the bipartite geminivirus nuclear shuttle protein. *Plant J.*, 5, 869-880.

Carvalho, M.F., Lazarowitz, S.G. (2004) Interaction of the movement protein NSP and the *Arabidopsis* acetyltransferase AtNSI is necessary for cabbage leaf curl geminivirus infection and pathogenicity. *J. Virol.* 78, 11161–11171.

Carvalho, C.M., Machado, J.P.B., Zerbini, F.M., Fontes, E.P.B. (2008b) NSP-interacting GTPase: A cytosolic protein as cofactor for nuclear shuttle proteins. *Plant Signal. Behav.* 3, 752-754.

Florentino, L.H., Santos, A.A., Fontenelle, M.R., Pinheiro, G.L., Zerbini, F.M., Baracat-Pereira, M.C., Fontes, E.P.B. (2006) A PERK-Like receptor kinase interacts with the geminivirus nuclear shuttle protein and potentiates viral infection. *J. Virol*. 80, 6648-6656.

Fondong, V.N. (2013) Geminivirus protein structure and function. *Mol. Plant Pathol*.14, 635–649.

Fontes, E.P.B., Gladfelter, H.J., Schaffer, R.L., Petty, I.T.D., Hanley-Bowdoin, L. (1994) Geminivirus replication origins have a modular organization. *Plant Cell.* 6, 405–416.

Fontes, E.P.B., Santos, A.A., Luz, D.F., Waclawovsky, A.J., Chory, J. (2004) The geminivirus nuclear shuttle protein is a virulence factor that suppresses transmembrane receptor kinase activity. *Genes Dev*. 18, 2545-2556.

Gouveia, B.C.C, Calil, I.P., Machado, J.P.B., Santos, A.A., Fontes, E.P.B. (2017) Immune Receptors and Co-receptors in Antiviral Innate Immunity in Plants. *Front. Microbiol.* 7, 2139.

Hanley-Bowdoin, L., Bejarano, E.R., Robertson, D., Mansoor, S. (2013) Geminiviruses: masters at redirecting and reprogramming plant processes. *Nat. Rev. Microbiol*. 11, 777–88.

Incarbone M., Dunoyer P. (2013) RNA silencing and its suppression: novel insights from in planta analyses. T*rends Plant Sci.* 18, 382–392.

Kørner, C.J., Klauser, D., Niehl, A., Dominguez-Ferreras, A., Chinchilla, D., Boller,T. *et al*. (2013)The immunity regulator BAK1 contributes to resistance against diverse RNA viruses. *Mol. Plant Microbe Interact.* 26, 1271–1280.

Machado, J.P.B., Brustolini, O.J.B., Mendes, G.C., Santos, A.A., Fontes, E.P.B. (2015) NIK1, a host factor specialized in antiviral defense or a novel general regulator of plant immunity? *Bioessays,* 37,1236–1242.

Machado, J.P.B., Calil, I.P., Santos, A.A., Fontes, E.P.B. (2017) Translational control in plant antiviral immunity*. Gen. Mol. Biol.*, 40, 1-13.

Mandadi, K.K., Scholthof, K-BG. (2013) Plant immune responses against viruses: how does a virus cause disease? *Plant Cell.* 25, 1489–505.

Mariano, A.C., Andrade, M.O., Santos, A.A., Carolino, S.M.B., Oliveira, M.L., Baracat-Pereira, M.C., Brommonshenkel, S.H., Fontes, E.P. (2004) Identification of a novel receptor-like protein kinase that interacts with a geminivirus nuclear shuttle protein. *Virol. J*. 318, 24-31.

Lazarowitz, S.G., Beachy, R.N. (1999) Viral movement proteins as probes for intracellular and intercellular trafficking in plants. *Plant Cell*. 11, 535–548.

Rojas, M.R., Hagen, C., Lucas, W.J., Gilbertson, R.L. (2005) Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. *Annu. Rev. Phytopathol*. 43, 361–394.

Scholthof, K.-B.G., Adkins, S., Czosnek, H., Palukaitis, P., Jacquot, E., Hohn, T., Hohn, B., *et al*., (2011) Top 10 plant viruses in molecular plant pathology. *Mol. Plant Pathol.* 12, 938–954.

Zhou, Y., Rojas, M.R., Park, M.R., Seo, Y.S., Lucas, W.J., Gilbertson, R.L. (2011) Histone H3 interacts and colocalizes with the nuclear shuttle protein and the movement protein of a geminivirus. *J. Virol*. 85, 11821–11832.

Zorzatto, C., Machado, J.P.B., Lopes, K.V.G., Nascimento, K.J.T., Pereira, W.A., Brustolini, O.J.B., *et al*., (2015) NIK1-mediated translation suppression functions as a plant antiviral immunity mechanism. *Nature.* 520, 679-682.

Chapter I

PLANT IMMUNITY AGAINST VIRUSES: ANTIVIRAL IMMUNE RECEPTORS IN FOCUS

Published paper

Calil, I. P., and Fontes, E. P. (2016). Plant immunity against viruses: antiviral immune receptors in focus. Ann. Bot. doi: 10.1093/aob/mcw200

Pages 9 to 22

Annals of Botany Page 1 of 13
doi:10.1093/aob/mcw200, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

REVIEW ARTICLE

Plant immunity against viruses: antiviral immune receptors in focus

Iara P. Calil and Elizabeth P. B. Fontes*

Departamento de Bioquímica e Biologia Molecular/National Institute of Science and Technology in Plant–Pest Interactions/Bioagro, Universidade Federal de Viçosa, 36570.000, Viçosa, MG, Brazil
**For correspondence. E-mail bbfontes@ufv.br*

Received: 9 February 2016 Returned for revision: 18 June 2016 Accepted: 5 August 2016

• **Background** Among the environmental limitations that affect plant growth, viruses cause major crop losses worldwide and represent serious threats to food security. Significant advances in the field of plant–virus interactions have led to an expansion of potential strategies for genetically engineered resistance in crops during recent years. Nevertheless, the evolution of viral virulence represents a constant challenge in agriculture that has led to a continuing interest in the molecular mechanisms of plant–virus interactions that affect disease or resistance.
• **Scope and Conclusion** This review summarizes the molecular mechanisms of the antiviral immune system in plants and the latest breakthroughs reported in plant defence against viruses. Particular attention is given to the immune receptors and transduction pathways in antiviral innate immunity. Plants counteract viral infection with a sophisticated innate immune system that resembles the non-viral pathogenic system, which is broadly divided into pathogen-associated molecular pattern (PAMP)-triggered immunity and effector-triggered immunity. An additional recently uncovered virus-specific defence mechanism relies on host translation suppression mediated by a transmembrane immune receptor. In all cases, the recognition of the virus by the plant during infection is central for the activation of these innate defences, and, conversely, the detection of host plants enables the virus to activate virulence strategies. Plants also circumvent viral infection through RNA interference mechanisms by utilizing small RNAs, which are often suppressed by co-evolving virus suppressors. Additionally, plants defend themselves against viruses through hormone-mediated defences and activation of the ubiquitin–26S proteasome system (UPS), which alternatively impairs and facilitates viral infection. Therefore, plant defence and virulence strategies co-evolve and co-exist; hence, disease development is largely dependent on the extent and rate at which these opposing signals emerge in host and non-host interactions. A deeper understanding of plant antiviral immunity may facilitate innovative biotechnological, genetic and breeding approaches for crop protection and improvement.

Key words: Antiviral immunity, antiviral immune receptors, PAMP-triggered immunity, effector-triggered immunity, NSP-interacting kinase, NIK-mediated translation suppression, antiviral RNA silencing, hormone-mediated defence, proteasome degradation, NBS-LRR resistance protein, receptor-like kinase, LRR-RLK.

INTRODUCTION

As obligate parasites with limited viral genome-encoded functions, plant viruses extensively use the host intracellular machinery for replication of their genomes, expression of viral genes and establishment of infection. As a consequence, they interact profoundly with the host during their biological cycle. In contrast to animal viruses, which use host surface receptors and endocytic activities to invade host cells, plant viruses are delivered into the cells by insect vectors or through opportunistic mechanical wounds. Once inside the cells, the viral particles, which minimally consist of nucleic acids encapsulated by the coat protein or capsid, are disassembled to release the viral genome and to initiate the infectious cycle, which includes expression and replication of the viral genome, cell to cell and long-distance movement of the viral particles and/or viral genome and vector-mediated transmission to new hosts. The extensive interactions between plant viruses and their hosts during infection lead to the physiological disorders responsible for plant diseases, which represent major constraints to agricultural productivity worldwide.

Plants employ multiple defence mechanisms to restrict viral replication and movement, such as gene silencing, immune receptor signalling, hormone-mediated defence, protein degradation and regulation of metabolism (Incarbone and Dunoyer, 2013). In virus–plant interactions, one of the major mechanisms for plant antiviral immunity relies on RNA silencing, which is often suppressed by co-evolving viral suppressors, thus enhancing viral pathogenicity in susceptible hosts. In addition, plants use nucleotide-binding leucine-rich repeat (NB-LRR) domain-containing resistance proteins, which recognize viral effectors and activate effector-triggered immunity (ETI) in a defence mechanism similar to that employed in non-viral infections (Mandadi and Scholthof, 2013). Plants have also been found to use innate pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) to limit viral infection (Kørner *et al*., 2013). More recently, a transmembrane immune receptor, which is structurally similar to co-receptor-like kinases involved in PTI, has been shown to activate host translation suppression to fight DNA viruses, a newly discovered mechanism for antiviral defences in plants (Zorzatto *et al*., 2015).

Viral infections can also lead to hormonal disruption, which manifests as simultaneous induction of many antagonistic hormones and triggering of defence responses (Alazem and Lin, 2015). Virus–host interactions can aberrantly regulate phytohormone pathways, leading to disease development and hormone-mediated defensive responses. Plants employ the ubiquitin–proteasome pathway (UPS) as an antiviral defence strategy and, concomitantly, viruses have been reported to exploit the UPS to induce, inhibit or modify ubiquitin (Ub)-related host proteins (Alcaide-Loridan and Jupin, 2012). In this review, we summarize recent reports on host–virus interactions, highlighting mechanisms adopted by plants to overcome viral infections in a continuous coevolutionary race for dominance. A major focus is antiviral immune receptors and their signal transduction pathways.

PLANT INNATE IMMUNE SYSTEM: DETECTION AND SIGNALLING IN ANTIVIRAL DEFENCES

Effector-triggered immunity: intracellular immune receptor R (resistance protein) in virus–plant interactions

The plant innate immune pathway employs a two-level detection system, which involves plasma membrane-localized and intracellular immune receptors, to activate defences against invaders (Dodds and Rathjen, 2010; Zipfel, 2014). In the first level of defence, PTI is mediated by surface-localized pattern recognition receptors (PRRs), which detect and recognize PAMPs (Böhm *et al*., 2014; Macho and Zipfel, 2014). The second level, ETI, involves intracellular immune receptors, designated as resistance proteins (R), which recognize – directly or indirectly – virulence effectors secreted by the pathogens into the host intracellular environment, thereby activating a defence response (Jones and Dangl, 2006) (Fig. 1).

The tobacco *N* gene was the first-identified *R* gene, which confers resistance against the *Tobacco mosaic virus* (TMV) (Whitham *et al*., 1994). Since then, many R genes involved in antiviral resistance in plants have been identified (Gururani *et al*., 2012; Mandadi and Scholtor, 2013), such as *Sw-5* for *Tomato spotted wilt virus* (TSWV) in tomato (Brommonschenkel *et al*., 2000), *Rx1* and *Rx2* for *Potato virus X* (PVX) in potato (Bendahmane *et al*., 1999, 2000), *RTM1* and *RTM2* for *Tobacco etch virus* (TEV), *RCY1* for *Cucumber mosaic virus* (CMV) in arabidopsis (Chisholm *et al*., 2000; Whitham *et al*., 2000; Takahashi *et al*., 2001) and the I locus for *Bean common mosaic virus* (Vallejo *et al*., 2006). A majority of the known R proteins belong either to the coiled-coil (CC)-NB-LRR or Toll/interleukin-1 receptor (TIR)-NBS-LRR class (Zhu *et al*., 2013; for a further review, see Gururani *et al*., 2012).

The *Rx* gene from potato, which encodes an NBS-LRR-type protein with a CC domain at the N-terminus (CC-NBS-LRR), may be the best-characterized resistance gene in plant–virus interactions (Bendahmane *et al*., 1999). The Rx N-terminal CC domain interacts intramolecularly with the Rx NB-LRR region and intermolecularly with the Rx cofactor RanGAP2 (Ran GTPase-activating protein 2) (Rairdan *et al*., 2008; Tameling *et al*., 2010). The C-terminus of the LRR domain is also thought to be involved in the specific recognition of the viral effector, which is functionally represented by the coat protein (CP), although a direct interaction between the CP and Rx has not been demonstrated (Bendahmane *et al*., 1995; Candresse *et al*., 2010; Dangl and Jones, 2001; Farnham and Baulcombe, 2006). The current mechanistic model for Rx function predicts that Rx is activated upon recognition of the Ran GTPase-mediated interaction with the CP.

Tobacco N protein represents a well-characterized example of the TIR-NBS-LRR class of R proteins in plant–virus interactions. The N resistance protein directly interacts with the helicase domain of the TMV replicase to trigger resistance (Ueda *et al*., 2006). Full resistance to TMV, however, depends on the N receptor-interacting protein 1 (NRIP1), which is recruited from the cytoplasm to the cytosol and nucleus to interact directly with both the N resistance protein and TMV replicase (Caplan *et al*., 2008). In both Rx-mediated resistance and N-mediated resistance, the R protein is activated in the cytoplasm, but full functionality of the Rx and N resistance proteins depends on their nucleocytoplasmic distribution. The R signalling cascade in plant–virus interactions consists of rapid activation of mitogen-activated protein kinases (MAPKs) and the involvement of molecular chaperone complexes controlling R protein stabilization and destabilization (Kadota and Shirasu, 2012; Hoser *et al*., 2013).

Generally, in plant–pathogen interactions, the immune responses downstream of R protein activation are associated with reactive oxygen species (ROS) production, calcium ion influx, MAPK activation, salicylic acid (SA) accumulation and massive transcriptional reprogramming, including the induction of genes associated with defence responses. Frequently, as in the case of N-mediated resistance, R protein activation also leads to the induction of a hypersensitive response (HR), which is often associated with programmed cell death of the infected and adjacent cells, confining the pathogen to the local site of infection. Concomitantly with the induction of the local defence response, R protein activation also activates defence signalling at distal tissues of infection, referred to as systemic acquired resistance (SAR), a defence mechanism shared by both Rx-mediated resistance and N-mediated resistance and induced by SA accumulation. More detailed information on SA signalling in defence is discussed in the hormone-mediated defence section.

Well-characterized exceptions to the NBS-LRR configuration of R proteins include the non-NBS-LRR-encoding *RTM* genes, which confer dominant resistance to TEV, *Lettuce mosaic potyvirus* (LMV) and *Plum pox potyvirus* (PPV) (Cosson *et al*., 2012), and the tomato *Tm-1* gene, which encodes a protein with a TIM-barrel-like structure and confers dominant resistance to TMV. The *Tm-1*-encoded product interacts directly with the viral replicase, impairing viral genome replication (Ishibashi and Ishikawa, 2013).

Recessive resistance

In addition to dominant R genes, recessive R genes have also been reported, and most of them confer resistance against viruses (Kang *et al*., 2005). A compatible virus–host interaction leading to systemic infection requires replication of the virus genome in addition to cell to cell and long-distance movement through the plant vascular system. Disruption of any of these

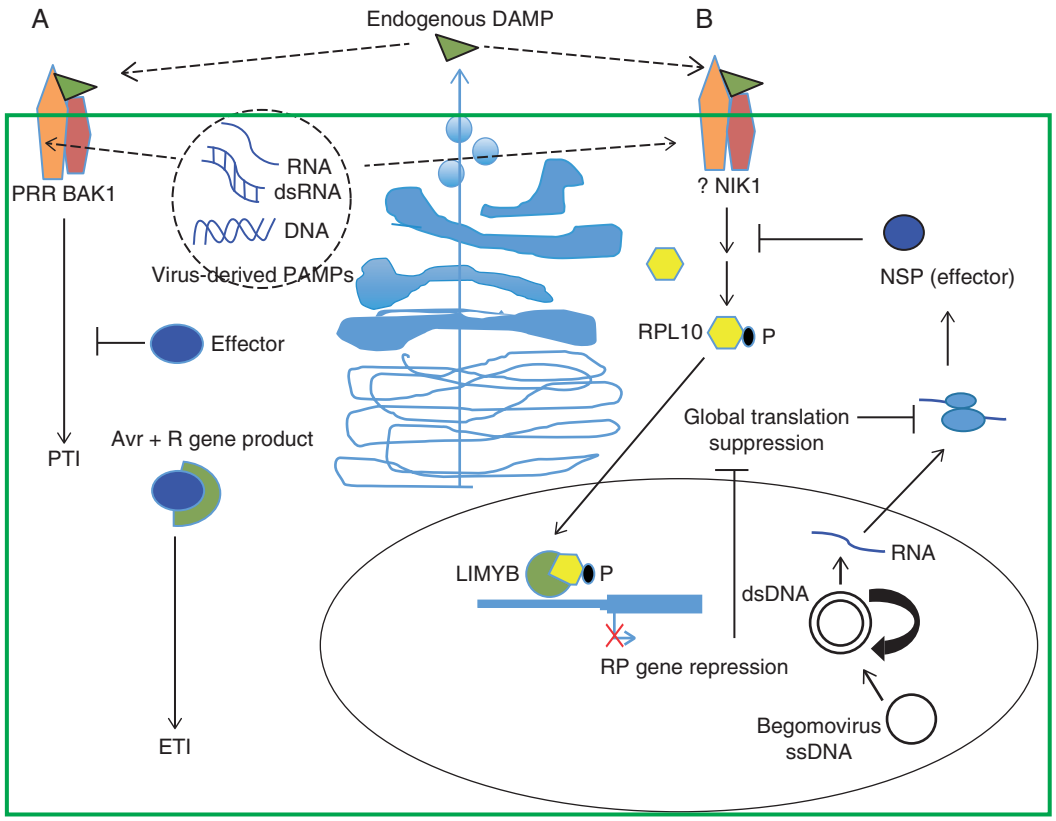

FIG. 1. Antiviral innate immunity in plants. (A) PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) in virus–host interactions. During viral infection, the replication and expression of the viral genome lead to the accumulation of virus-derived nucleic acids with features of pathogen-associated molecular patterns (PAMPs), which may be recognized by host pattern recognition receptors (PRRs) that, in turn, heterooligomerize with co-receptors, such as BAK1 and BKK1, to trigger PTI. Alternatively, PTI may be activated upon PRR recognition of damage-associated molecular patterns (DAMPs), which are induced by infection and delivered to the apoplast by the host cells via the secretory apparatus. In a successful infection, expression of the viral genome results in accumulation of virus effectors to suppress PTI, leading to disease. In resistant genotypes, however, the resistance genes specifically recognize, directly or indirectly, the viral effectors, called avirulence (Avr) factors, activating ETI and conferring resistance. (B) The translational control arm of the NIK1-mediated signalling in antiviral innate immunity. Virus infection-induced oligomerization of NIK1 promotes transphosphorylation at the crucial Thr474, activating the kinase. Alternatively, NIK1 interacts with an unknown ligand-binding LRR-RLK in a stimulus-dependent manner. Although viral infection triggers NIK1-mediated antiviral signalling, the molecular basis of this elicitation is unknown and may be either intracellular virus-derived nucleic acid PAMPs or endogenous DAMPs released in the apoplasts by the host cells. Upon activation, NIK1 indirectly mediates the RPL10 phosphorylation, promoting its translocation to the nucleus, where it interacts with LIMYB to down-regulate the expression of translation-related genes. Therefore, the propagation of the antiviral signal culminates with suppression of host global protein synthesis, which also impairs translation of viral mRNA, as a defence mechanism. In begomovirus–host compatible interactions, the binding of begomovirus NSP to the NIK1 kinase domain (A-loop) inhibits autophosphorylation at Thr474, thereby preventing receptor kinase activation and RPL10 phosphorylation, overcoming this layer of defence. The viral single-stranded DNA replicates via double-stranded DNA intermediates that are transcribed in the nucleus of plant-infected cells. NSP binds to the nascent viral DNA and facilitates its movement to the cytoplasm and acts in concert with the classical movement protein MP to transport the viral DNA to the adjacent, uninfected cells.

processes results in incompatible interactions, which is often mediated by host resistance factors. The recessive gene-encoded products are involved in compatibility functions; they are not immune receptors and are not associated with the ETI but rather act as essential factors required for the virus to complete its biological cycle. Therefore, many plant natural resistance genes have been mapped to mutations of essential host factors for virus infection. Examples of recessive resistance genes include eukaryotic translation initiation factors, such as eIF4E and eIF4G, which play an essential role in successful infection by potyviruses, bymoviruses, cucumoviruses, ipomoviruses, sobemoviruses, carmoviruses and waikiviruses, and thereby resistance is conferred by *eIF4E* and *eIF4G* loss-of-function mutations or modification of their gene products (Revers and Nicaise, 2014).

Antiviral immune receptors in PAMP-triggered immunity

The first layer of innate immunity is immediately activated upon host detection of highly conserved structural motifs exclusively expressed by pathogens, known as PAMPs, or endogenous danger signals released by the host during a wound or pathogenic attack known as damage-associated molecular patterns (DAMPs), which function as elicitors (Macho and Zipfel, 2014). The recognition of different PAMPs or DAMPs by specific cell surface sensors, designated PRRs, activates a sophisticated defence signalling cascade which inhibits a broad spectrum of potential pathogens, including bacteria, viruses, fungi and oomycetes. In plants, the PRRs are represented by receptor-like kinases (RLKs) and receptor-like proteins (RLPs) located at the cell surface. Both RLKs and RLPs often require a

co-receptor to form an active complex to initiate signalling. The best-characterized co-receptor in PTI is the BRASSINOSTEROID INSENSITIVE1 (BRI1)-associated kinase 1, BAK1, which forms active signalling complexes with both RLKs and RLPs after PAMP detection by PRRs (Liebrand *et al*., 2014; Postma *et al*., 2016). BAK1 belongs to the LRR-RLK family and has an N-terminal extracellular LRR domain, which is structurally similar to mammalian Toll-like receptor (TLR) immune sensors, a transmembrane segment and an intracellular kinase domain. BAK1 heterodimerizes with several LRR-RLK immune sensors, including FLS2 (flagellin receptor), EFR (bacterial elongation factor-Tu receptor) and PEPR1 (damage-associated peptide 1 receptor), and is functionally required in immunity and signalling triggered by multiple bacterial PAMPs. The BAK1 positive regulation in plant immunity involves phosphorylation reactions between the BAK1 co-receptor and the corresponding PRR.

In the case of viral pathogens, the innate immune system has been primarily described in mammalian cells, which often detects specific biochemical features that are exclusive to the viral nucleic acid genome. Viral genomes exist as single- or double-stranded RNA or DNA and can be monopartite or partitioned into two or more segments. In mammalian cells, the TLRs comprise a large family of nucleic acid-sensing PRRs, which have relevant roles in antiviral defence. TLRs are similar to LRR-RLKs; they are single, membrane-spanning receptors with an LRR extracellular domain. Different members of the TLR family recognize different biochemical features present in viral, but not in host, nucleic acids, such as single-stranded RNA without a 5′ cap, double-stranded RNA (dsRNA) or unmethylated DNA. Specific recognition also relies on the opportunistic subcellular localization of TLRs and the viral genome in host cells. Although specific PRRs for viral recognition have not yet been found in plants, accumulated data indicate that plant PTI signalling inhibits viral infection similarly to non-viral pathogens. In fact, plant–virus interactions induce a complex set of typical PTI responses, including ROS production, ion fluxes, SA accumulation, defence gene activation, such as PR-1, and callose deposition (for a review, see Nicaise, 2014). In addition, upstream and downstream components of the PTI signalling pathway have been shown to play a role in antiviral defence. The functions of the PTI co-receptors BAK1 and BKK1 (BAK1-like kinase 1) are required to build an effective defence against RNA viruses in arabidopsis (Yang *et al*., 2010; Kørner *et al*., 2013), and MAPK4, a negative regulator of plant PTI signalling, suppresses soybean defence against *Bean pod mottle virus* (BPMV; Liu *et al*., 2011). Furthermore, the pre-activation of PTI by the elicitor chitosan, through interaction with chitin-binding PRRs, has also been shown to be effective against viruses (Iriti and Varoni, 2014). Finally, according to the zigzag evolutionary model of plant innate immunity (Jones and Dangl, 2006), the involvement and activation of ETI in plant–virus interactions is conceptually associated with successful PTI inhibition by a viral effector, further substantiating the argument that an antiviral PTI mechanism operates in plants as well (Fig. 1). Given the mode of virus delivery into plant cells and the obligatory conservative nature of PAMPs, which is not a property of rapidly evolving plant virus proteins, the molecular nature of the virus signatures recognized by plant PTI is very probably similar to those presented by mammalian viruses during infection. Therefore, the discovery of plant antiviral PRRs is expected to accelerate the characterization of nucleic acid-sensing PRRs and/or DAMP-sensing PRRs in plants.

Immune receptor-mediated suppression of translation: a new paradigm for antiviral defences in plants

NIK1 as an antiviral immune receptor. The immune receptor NIK1 [nuclear shuttle protein (NSP)-interacting kinase 1], a RLK family member, has a remarkable role in the defence response against geminiviruses (Fontes *et al*., 2004). Although NIK1 shows structural similarities to BAK1, the mechanism for NIK-mediated antiviral defence is completely different from classical BAK1-mediated PTI (Machado *et al*., 2015).

NIKs (NIK1, NIK2 and NIK3) were first identified as targets of the NSP from *Begomovirus*, the largest genus of the *Geminiviridae* family (Fontes *et. al*., 2004). The NSP–NIK interaction is conserved among begomovirus NSPs and NIK homologues from different hosts (Mariano *et al*., 2004). NIK homologues from arabidopsis, tomato and soybean interact with NSPs from *Cabbage leaf curl virus* (CaLCuV) and from tomato-infecting begomoviruses, such as *Tomato golden mosaic virus* (TGMV), *Tomato crinkle leaf yellow virus* (TCrLYV) and *Tomato yellow spot virus* (ToYSV) (Fontes *et al*., 2004; Mariano *et al*., 2004; Sakamoto *et al*., 2012). These interactions suppress the NIK kinase activity and prevent the activation of the antiviral signal transduction pathway, creating a suitable environment for begomovirus infection (Santos *et al*., 2009, 2010). Consistent with a role for NIK in antiviral defence, loss-of-function *nik1*, *nik2* and *nik3* mutants showed enhanced susceptibility to CaLCuV infection (Fontes *et al*., 2004; Rocha *et al*., 2008; Santos *et al*., 2009). In addition, overexpression of NIK1 delays viral infection and attenuates symptom development in tomato (Carvalho *et al*., 2008). Finally, mutations in the activation loop (A-loop) of NIK1 that prevent its autophosphorylation also compromise the capacity of NIK1 to elicit a response against begomoviruses (Santos *et al*., 2009).

Mechanisms of NIK1 activation. As a single-pass transmembrane receptor kinase, NIK is expected to dimerize or multimerize with itself and/or co-receptors to promote transphosphorylation and subsequent activation of the kinase. However, there is a complete lack of information on the critical early event that triggers NIK1 signalling and transduction, which culminates with suppression of host global translation as an antiviral response. Recently, a comparison between the transcriptomes induced by begomovirus infection and expression of a constitutively activated NIK1 receptor revealed that begomovirus infection is the activating stimulus of NIK1-mediated defence, although the molecular basis for this elicitation is still unknown. By comparison with the mechanism of mammalian antiviral immune receptor activation, one can predict that unique biochemical features of the begomovirus genome function as possible ligands that trigger or stabilize NIK dimerization or multimerization with a co-receptor. Begomoviruses are single-stranded DNA viruses, which replicate via double-stranded DNA intermediates in the nuclei of infected cells. The divergent transcription units of the viral genome result in single-stranded transcripts and double-stranded overlapping

RNAs as possible sources for specific nucleic acid ligands. In mammals, the cytoplasmic receptor PKR (protein kinase receptor), which is activated by dsRNA molecules of > 40 bp, mediates global translation suppression by phosphorylating eIF2α on Ser51 as an antiviral response (Jackson *et al*., 2010). Alternatively or additionally, NIK1 activation may depend on host molecular signatures (DAMPs) released in the apoplast in response to viral infection.

Activation of many kinases requires phosphorylation of the activation segment (A-loop) that is defined by the region delimited by two conserved tripeptide motifs, DFG and APE (Nolen *et al*., 2004). This region is highly conserved among members of the LRR-RLK II subfamily and other members of the extended LRR-RLK family. The phosphorylation status of the activation segment has been shown to dictate NIK1 kinase activity (Carvalho *et al*., 2008; Fontes *et al*., 2004; Santos *et al*., 2009). NIK1 is phosphorylated *in vitro* at the conserved positions Thr474 and Thr469, and mutations in the A-loop compromise its autophosphorylation capacity (Santos *et al*., 2009). Replacement of Thr474 with alanine strongly inhibits the autophosphorylation activity and the capacity of NIK1 to elicit a defence response, whereas replacement of Thr474 with a phosphomimetic aspartate residue increases autophosphorylation activity and results in constitutive activation of a NIK1 mutant receptor that it is no longer inhibited by begomovirus NSP (Santos *et al*., 2009). These results indicate that phosphorylation at the essential Thr474 residue in the A-loop constitutes a key regulatory mechanism for NIK activation.

Although replacement of the essential Thr474 residue with the aspartate residue bypasses the NSP inhibitory effect on kinase activity, it does not impair NSP binding to an 80 amino acid stretch (positions 422–502) of NIK that encompasses the putative active site for Ser/Thr kinases (sub-domain VIb–HrDvKssNxLLD) and the activation loop (sub-domain VII–DFGAk/rx, plus sub-domain VIII–GtxGyiaPEY; Fontes *et al*., 2004). These results suggest that the NSP inhibitor acts upstream of the phosphorylation at position 474.

While phosphorylation at Thr474 is linked to an activation loop-dependent mechanism for NIK function, phosphorylation of Thr469 appears to have an autoinhibitory role (Santos *et al*., 2009). Replacing Thr469 with alanine relieves repression and enhances substrate phosphorylation. Furthermore, mutation at Thr469 does not inhibit autophosphorylation activity or impair the capacity of the mutant protein to elicit a defence response and to redirect the downstream component RPL10 to the nucleus. It has been proposed that autophosphorylation of Thr469 within the NIK1 A-loop allows the kinase to control the sustained signalling more efficiently. Whether this inhibitory mechanism allows NIK1 to phosphorylate pathway components differentially remains to be determined.

Downstream components of the NIK-mediated antiviral response. A ribosomal protein, RPL10, identified as a binding partner for NIKs, acts as a downstream effector of the NIK-mediated antiviral response. Arabidopsis *rpl10* mutants showed enhanced susceptibility to geminivirus infection, recapitulating the *nik1* phenotype (Rocha *et al*., 2008). Ectopic expression of NIK1 or a hyperactive NIK1 mutant led to relocation of phosphorylated RPL10A from the cytosol to the nuclei (Carvalho *et al*., 2008). In addition, an inactive NIK1 mutant failed to redirect the protein to the nuclei of co-transfected cells, while a mutant RPL10A defective for NIK1 phosphorylation is not redirected to the nucleus and does not mount a defence response against begomoviruses. These data suggest that the nucleocytoplasmic shuttling of RPL10 is regulated by phosphorylation and is dependent on the kinase activity of NIK1, classifying RPL10 as a downstream effector of NIK1-mediated signalling.

Although RPL10 binds to NIK1 *in vitro* and *in vivo*, it is not efficiently phosphorylated by NIK1 *in vitro* and may not serve as a direct NIK1 substrate *in vivo*. Nevertheless, the nucleocytoplasmic shuttling of RPL10 is regulated by phosphorylation and is dependent on the kinase activity of NIK1. In fact, NIK1 does not relocate a phosphorylation-deficient mutant of RPL10 to the nucleus (Carvalho *et al*., 2008). Furthermore, the gain-of-function T474D mutant is more effective at redirecting RPL10 to the nucleus, and inactive mutants of NIK1 fail to alter the cytosolic localization of RPL10 (Santos *et al*., 2009). Mutations in the A-loop similarly affect the capacity of NIK1 to elicit an antiviral response and to mediate the phosphorylation-dependent nuclear relocalization of RPL10.

In order to gain new insights into the molecular mechanisms of NIK1 in antiviral immunity, arabidopsis transgenic lines harbouring the gain-of-function mutant T474D on a *nik1* knockout background were analysed for gene expression (Zorzatto *et al*., 2015). The constitutive activation of NIK-mediated signalling resulted in the down-regulation of translation-related genes and the suppression of global translation, decreasing the loading of host mRNAs in actively translating polysomes (Zorzatto *et al*., 2015). In begomovirus-infected lines, the association of viral mRNA with actively translating polysomes was lower in T474D lines than in the wild type, indicating that the begomovirus is not capable of sustaining high levels of viral mRNA translation when global host translation is impaired. Accordingly, the transgenic lines ectopically expressing T474D displayed enhanced resistance to begomovirus, demonstrating that suppression of global protein synthesis may effectively protect plant cells against DNA viruses.

Further analyses detected LIMYB, an RPL10-interacting MYB domain-containing transcriptional factor, as another downstream component of the NIK1-mediated antiviral pathway (Zorzatto *et al*., 2015). LIMYB binds to and acts in concert with RPL10 to repress fully the expression of ribosomal gene expression. LIMYB overexpression represses ribosomal protein (RP) genes at the transcriptional level, resulting in protein synthesis inhibition, decreased viral mRNA association with polysome fractions and enhanced tolerance to the begomovirus CaLCuV. In contrast, loss of LIMYB function releases repression of RP genes and recapitulates the enhanced susceptibility phenotype of the *nik1* null alleles. T474D also downregulates the expression of the same sub-set of LIMYB-regulated RP genes but requires LIMYB to repress RP gene expression. Therefore, LIMYB is a downstream transcriptional repressor in the NIK1-mediated pathway, which links NIK1 activation to the downregulation of translational machinery-related genes, thereby suppressing global host translation as an antiviral immunity strategy in plants.

NIK1-mediated translation suppression may act as a conserved antiviral mechanism in begomovirus–host interactions. Tomato T474D transgenic lines were tolerant to the tomato-infecting begomoviruses ToYSV and ToSRV (*Tomato severe*

rugose virus), which display highly divergent genomic sequences and hence are only distantly related within the group of tomato-infecting begomoviruses (Brustolini *et al*., 2015). As in arabidopsis-infected T474D lines, overexpression of T474D in tomato represses RP genes, suppresses global protein synthesis and decreases viral mRNA association with the polysome fractions (Brustolini *et al*., 2015). Therefore, the enhanced tolerance to tomato-infecting begomovirus displayed by the T474D-expressing lines is associated with the translational control branch of the NIK-mediated antiviral responses. These observations underscore the potential of a sustained NIK-mediated defence pathway to confer broad-spectrum tolerance to begomoviruses in distinct plant species. However, whether NIK-mediated suppression of global translation functions against plant RNA viruses it is still a matter of debate.

Mechanistic model for the NIK1-mediated antiviral signalling pathway. Since the discovery of NIKs, several features of the NIK1-mediated antiviral signalling and its interaction with the begomovirus NSP have been elucidated (Fig. 1). We now know that the transmembrane receptor NIK1, a serine/threonine kinase transducer, is activated by viral infection to trigger a defence response against the virus itself, although the molecular basis for this elicitation remains unknown. Based on common features of the LRR-RLKII family, we propose that the extracellular domain of NIK undergoes oligomerization with itself or with an unidentified ligand-dependent LRR-RLK receptor following viral infection. The ligand may be DAMPs delivered into the apoplast by the secretory apparatus upon detection of viral infection. Alternatively, NIK1 may recognize virus-derived nucleic acids as PAMPs that promote oligomerization of the antiviral immune receptor. Regulation of NIK kinase activity depends on a conformational change of the A-loop induced by phosphorylation of Thr474. Activated NIK regulates the nucleocytoplasmic trafficking of RPL10, which in turn interacts with the transcriptional repressor LIMYB to downregulate RP genes, leading to suppression of host and viral mRNA translation, thereby linking the antiviral response to receptor activation.

Nuclear shuttle protein prevents activation of the pathway by binding to the NIK kinase domain and sterically interfering with phosphorylation of Thr474 in the A-loop. As a consequence, phosphorylation of RPL10 is impaired, and the RP is trapped in the cytoplasm during begomovirus infection. NSP inhibition of NIK1 prevents activation of the NIK-mediated signalling pathway, resulting in an intracellular environment that is more favourable for viral proliferation and spread. The viral single-stranded DNA replicates via double-stranded DNA intermediates that are transcribed in the nucleus of plant-infected cells (Hanley-Bowdoin *et al*., 2013). NSP binds to the nascent viral DNA and facilitates its movement to the cytoplasm, acting in concert with the classical movement protein MP to transport the viral DNA to the adjacent, uninfected cells.

RNA SILENCING MACHINERY: AN ADAPTIVE ANTIVIRAL IMMUNITY MECHANISM

The RNA silencing pathway or RNA interference (RNAi) is a well-established natural antiviral defence mechanism in plants, in which the viruses are both inducers and targets of RNA silencing (Wang *et al*., 2010; Szittya *et al*., 2013). To inhibit RNA silencing, well-adapted plant viruses are known to encode silencing-suppressor proteins, which can counteract the host silencing-based antiviral process (Wieczorek and Obrepalska-Steplowska, 2015). In this review, we summarize the conceptual advances in the antiviral RNAi mechanism and the evolving virulence strategies to overcome this adaptive plant defence (Fig. 2). For more detailed information, a collection of excellent, updated reviews describing antiviral RNA silencing mechanisms and suppressors is available (Carbonell and Carrington, 2015; Csorba *et al*, 2015; Zhang *et al*., 2015).

The diversity in RNAi mechanisms relies mainly on the existence of multiple copies of AGO (Argonaute), RNA-dependent RNA polymerase (RDR), DRB (double-stranded RNA binding) and DCL (Dicer-like) genes, which probably result from gene duplication followed by specialization (Parent *et al*., 2015; Zhang *et al*., 2015). DCL2 and DCL4 have a crucial role in antiviral defence (Deleris *et al*. 2006; Qu *et al*., 2008; Garcia-Ruiz *et al*., 2010). Arabidopsis plants containing loss-of-function mutations within the Dicer-like 2 (DCL2), Argonaute 2 (AGO2) and HEN1 RNA methyltransferase were more susceptible to *Turnip crinkle virus* (TCV) infection (Zhang *et al*., 2012). Arabidopsis *dlc4* mutants inoculated with TCV lacking P38 (silencing suppressor) exhibited large primary lesions, but viral systemic movement was compromised. However, viral infection was fully established in *dcl2–dcl4* double mutants (Deleris *et al*., 2006). Recently, Andika *et al*. (2015) demonstrated the differential requirement for the DCL4 and DCL2 proteins in the inhibition of intracellular and systemic infection, respectively, by PVX in arabidopsis, which highlights the host's ability to fight against both local and systemic viral infection.

Although DCL3 has a minor role against RNA viruses, it is crucial against DNA viruses (Qu *et al*., 2008; Csorba *et al*., 2015). Arabidopsis *dcl3* mutants are unable to recover from geminivirus infection, while remission was observed in wild-type, *dcl2* and *dcl4* plants (Raja *et al*., 2014). Plants employ RNA-directed DNA methylation (RdDM) as an epigenetic defence against geminiviruses (Raja *et al*., 2008, 2014; Ruiz-Ferrer and Voinnet, 2009; Hanley-Bowdoin *et al*., 2013). Arabidopsis methylation-deficient mutants are hypersusceptible to geminivirus infection. Additionally, cytosine methylation levels are significantly reduced in viral DNA isolated from methylation-deficient mutants (Raja *et al*., 2008). DCLs interact with DRBs to produce small RNAs. The DRB3 protein functions with Dicer-like 3 (DCL3) and Argonaute 4 (AGO4) in methylation-mediated antiviral defence (Raja *et al*., 2014). In turn, some DNA viruses can suppress silencing by interfering with the methyl cycle. A silencing suppressor from begomovirus, AC2, inhibits host adenosine kinase (ADK) activity, which is required for RNA silencing (Wang *et al*., 2003, 2005). The AL2-mediated silencing suppression was followed by reduced cytosine methylation (Buchmann *et al*., 2009). The betasatellite-encoded protein, βC1, from *Tomato yellow leaf curls China virus* (TYLCCNV) also targets the methyl cycle through inhibition of *S*-adenosylhomocysteine hydrolase (SAHH) activity (Yang *et al*., 2011). Geminiviruses also employ an alternative mechanism to interfere with the host DNA methylation machinery during the infection by reducing the transcript levels of Methyltransferase 1 (MET1) and

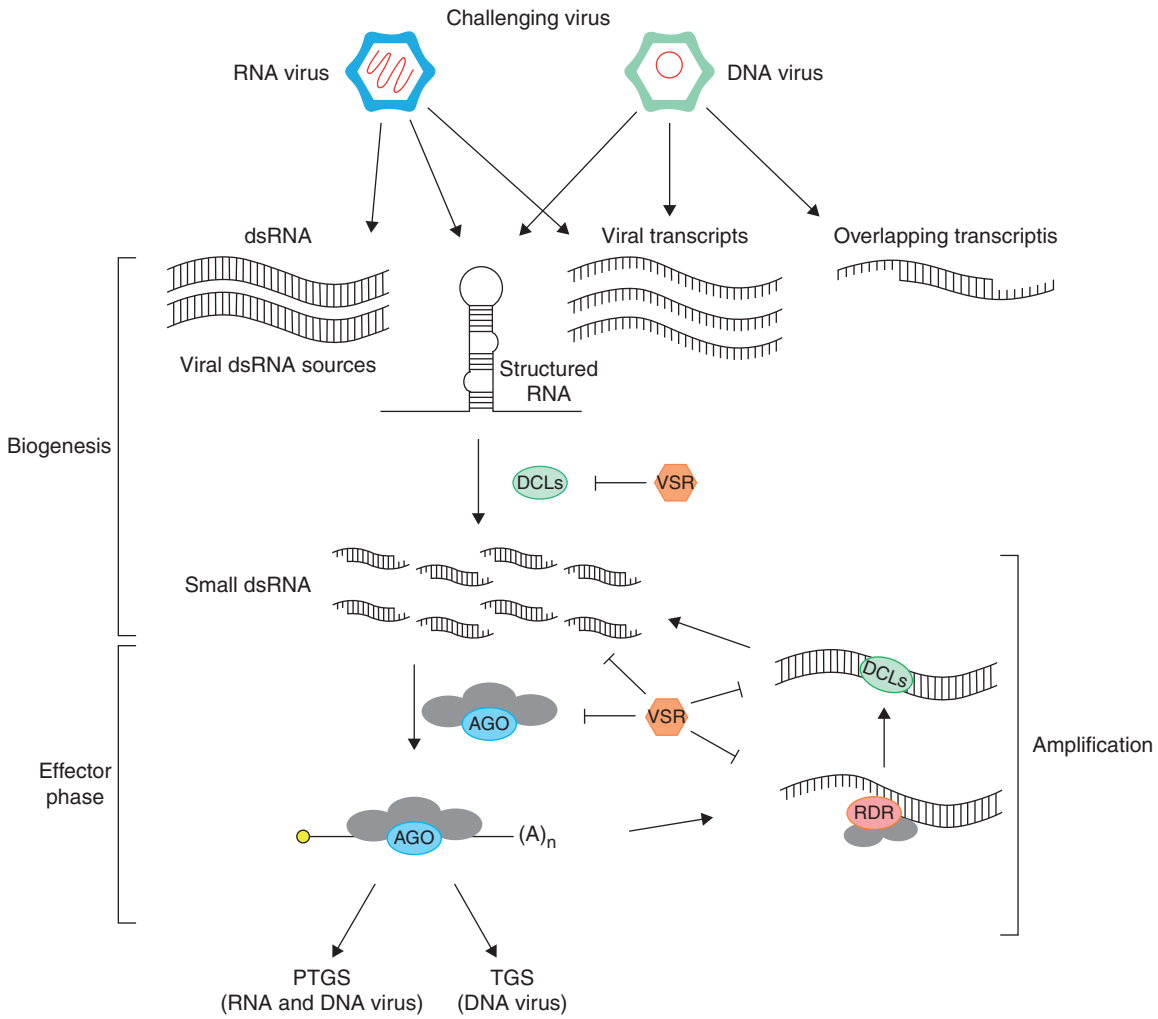

FIG. 2. Adaptive antiviral immunity in plants: general model of antiviral RNA silencing and its suppression by viral suppressors of RNA silencing (VSRs). The Silencing response is triggered by viral dsRNA molecules (vsRNA, ds-siRNA, 21, 22 rr 24 nt) from different sources, which are produced by Dicer-like proteins (DCLs). These vsRNAs are subsequently loaded into Argonaute (AGO)-containing silencing complexes. In post-transcriptional gene silencing (PTGS), viral RNA is targeted by the RNA-induced silencing complex (RISC) for degradation or translational repression, while the RNA-induced transcriptional silencing complex (RITS) causes histone and/or DNA methylation, leading to transcriptional gene silencing (TGS). The effector phase can also result in the amplification of silencing response through the action of RNA-dependent RNA polymerase (RDR) proteins, which produce more dsRNA substrates for DCL processing. VSRs can target multiple steps of the RNA silencing pathway, defeating host antiviral mechanisms by interfering in dicing, vsRNA loading, AGO activation and amplification.

Chromomethylase 3 (CMT3), key enzymes of the plant methylation cycle (Rodriguez-Negrete *et al.*, 2013). The replicase-associated protein (Rep) is responsible for the repression of MET1 and CMT3, and another viral protein, C4, has an auxiliary role in MET1 down-regulation (Rodriguez-Negrete *et al.*, 2013).

The AGO proteins are essential in antiviral defence against both RNA and DNA viruses. AGO1, AGO2, AGO4, AGO5, AGO7 and AGO10 have been shown to display antiviral activity in arabidopsis, while AGO1 and AGO18 play antiviral defence roles in rice (reviewed in Carbonell and Carrington, 2015). RDR activities contribute to the amplification of antiviral activity. RDR1 and RDR6 play an essential role in the amplification of virus-derived small interfering RNAs (siRNAs; Wang *et al.*, 2010). The biogenesis of *Tobacco rattle virus* (TRV)-derived siRNAs involves the combined activity of RDR1, RDR2 and RDR6 (Donaire *et al.*, 2008). DCL4 and RDR1 are major contributors to biogenesis of *Turnip mosaic virus* (TuMV)-derived siRNAs, although a full antiviral defence also requires DCL2 and RDR6 (Gacia-Ruiz *et al.*, 2010). OsRDR6 knockdown transgenic rice show hypersusceptibility to *Rice stripe virus* (RSV). These phenotypes are associated with increased accumulation of RSV genomic RNA and reduced RSV-derived siRNA accumulation compared with the wild-type plants (Jiang *et al.*, 2012). Hong *et al.* (2015) also reported an increase in susceptibility to *Rice dwarf phytoreovirus* (RDV) in OsRDR6 downregulated rice followed by a reduction in the RDV vsiRNA levels. However, overexpression of OsRDR6 had no effect on RDV infection.

Many viral suppressor proteins can target multiple steps of the RNA silencing pathway to defeat host antiviral mechanisms. One strategy used by viral suppressors is impairment of

viral siRNA biogenesis by inhibiting DCL proteins and/or the activity of cofactors, sequestering dsRNA/siRNA or promoting AGO protein destabilization prior to RISC assembly (reviewed in Csorba *et al*., 2015). The p22 suppressor of *Tomato chlorosis virus* (ToCV) binds long dsRNAs *in vitro*, preventing them from being cleaved by an RNase III-type Dicer homologue, which might block the silencing process by interfering with the generation of siRNAs (Landeo-Rios *et al*., 2015). The silencing suppressor of *Lettuce necrotic yellows virus* (LNYV), phosphoprotein P, targets multiple proteins involved in the RNA silencing pathway, including those involved in the RISC complex and dsRNA amplification. LNYV P impairs RNA silencing through inhibition of micro RNA (miRNA)-guided AGO1 cleavage and translational repression and also compromises RDR6/SGS3-dependent amplification of silencing (Mann *et al*., 2016). One of the best-characterized suppressors of antiviral RNA silencing is the potyviral helper component proteinase (HCPro), which plays multiple roles in the suppression of vsiRNA biogenesis, such as ds–siRNA binding, HEN1 binding, blocking HEN1 methyltransferase activity, blocking primary siRNA biogenesis by RAV2 interaction and downregulating RDR6 (Zhang *et al*., 2015). A recent study suggested two mechanisms by which HCPro exerts its RNA silencing suppressor functions (Ivanov *et al*., 2016). HCPro may block siRNA methylation of HEN1 via inhibition of *S*-adenosyl-L-methionine synthase (SAMS) and SAHH, two key enzymes of the methionine cycle. HCPro may also attenuate viral RNA translational repression through association with AGO1 and ribosomes.

Over the past decades, significant advances have been made in the current understanding of the role of RNA silencing in plant antiviral immunity responses. Concomitantly, diverse mechanisms employed by viruses to avoid silencing-mediated resistance have been unravelled, most of them through silencing suppressor activities. Additionally, there are reports that plants have evolved specific defences against RNA silencing suppression. Collectively, these findings provide new insight into the molecular mechanisms mediating plant–virus interactions, and they concomitantly highlight a complex and lasting arms race between pathogens and their hosts.

HORMONE-MEDIATED ANTIVIRAL DEFENCES

Plant hormones play important roles in intercellular and systemic signalling systems, regulating developmental processes and plant responses to a wide range of biotic and abiotic stresses (Bari and Jones, 2009). In susceptible hosts, plant viruses often manipulate biochemical events and molecular interactions required for their replication and movement, leading to misregulation and disruption of hormone signalling (Alazem and Lin, 2015).

Salicylic acid is a key component of the plant response to pathogens and is involved in the establishment of local and systemic resistance (Vlot *et al*., 2009; Pieterse *et al*., 2012). The role of SA in viral defence was initially reported in the interaction between the TMV and the tobacco *N* resistance gene (Gaffney *et al*., 1993; Jovel *et al*., 2011). Tobacco transgenic lines deficient in SA accumulation were defective in their ability to induce SAR against TMV and inefficiently restricted virus movement (Gaffney *et al*., 1993). The SA pathway is typically activated by both DNA and RNA viruses (Whitham *et al*., 2006; Ascencio-Ibanez *et al*., 2008). Arabidopsis *cpr1* (constitutive expresser of PR genes) mutants, in which SA-mediated SAR is constitutively activated, were less susceptible to CaLCuV infection (Bowling *et al*., 1994). Additionally, the arabidopsis mutant *lsb1* (less susceptible to BSCTV 1) showed impairment in *Beet severe curly top virus* (BSCTV) DNA replication and reduced infectivity (Chen *et al*., 2010). Previous studies showed that upregulation of LSB1/GDU3 affects geminivirus infection by activating the SA pathway (Chen *et al*., 2010).

The SA defence response is also triggered by *Potato virus Y* (PVY) and *Tomato ringspot virus* (ToRSV) (Jovel *et al*., 2011; Baebler *et al*., 2014). The lack of SA accumulation in the NahG potato plants (transgenic lines deficient in SA accumulation) causes unrestricted viral spreading and consequent disease symptoms (Baebler *et al*, 2014). Transcriptomic analysis confirmed the central role of SA in inducing the *Ny-1*-mediated responses and showed that the absence of SA leads to significant changes at the gene expression level, including a delay in activation of defence genes. In a similar manner, SA-dependent mechanisms were implicated in the restriction of ToRSV spread in tobacco. Lesion size and viral systemic spread were reduced with SA pre-treatment but enhanced in NahG transgenic lines deficient in SA accumulation, (Jovel *et al*., 2011). The *eds5* (*enhanced disease susceptibility 5*) mutation and the *NahG* transgene partially defeated the resistance of *Col-24-C* to *Cucumber mosaic virus* strain-Y (CM-Y) (Takahashi *et al*., 2004).

Plum pox virus (PPV) replication is restricted to inoculated leaves in tobacco plants, but the virus is able to infect P1/HC-Pro-expressing plants systemically (Alamillo *et al*., 2006). Interestingly, PPV was also able to move systemically in NahG-expressing tobacco plants. Further analysis revealed reduced accumulation of viral-derived small RNAs in the NahG transgenic plants and enhanced expression of SA-mediated defence transcripts, such as those of pathogenesis-related (PR) proteins PR-1 and PR-2, alternative oxidase-1 and the putative RNA-dependent RNA polymerase NtRDR1, in response to PPV infection, suggesting that SA might act as an enhancer of RNA silencing in tobacco. SA treatments also induced resistance against TMV and activated the RNA silencing-related genes *DCL1*, *DCL2*, *RDR1* and *RDR2* in tomato plants (Campos *et al*., 2014).

The role of jasmonic acid (JA) signalling in virus defence is controversial. Genes involved in the JA pathway are generally suppressed during geminivirus infection (Ascencio-Ibanez *et al*., 2008). The viral pathogenesis factor βC1 from TYLCCNV attenuates expression of several JA-responsive genes (Yang *et al*., 2008). In contrast, multiple genes related to JA signalling were upregulated in transgenic tobacco plants expressing the viral silencing suppressor AC2 derived from *African cassava mosaic virus* (Soitamo *et al*., 2012). The AC2 protein also interacts with CSN5a, a COP9 signalosome component, interfering with the derubylation activity of the CSN complex and disturbing several cellular processes, including jasmonate responses (Lozano-Duran *et al*., 2011). Exogenous jasmonate treatment of *A. thaliana* plants disrupts geminivirus infection, suggesting that the suppression of the jasmonate response might be crucial for infection

(Lozano-Duran *et al*., 2011). In contrast, exogenously applied methyl jasmonate (MeJA) reduced local resistance to TMV and permitted systemic viral movement in *Nicotiana tabacum* (tobacco) cultivars while the silencing of CORONATINE-INSENSITIVE 1 (COI1), a JA receptor, reduced viral accumulation in a tobacco cultivar possessing the *N* gene, as did that of allene oxide synthase, a JA biosynthetic enzyme (Oka *et al*., 2013).

Brassinosteroids (BRs) have also been identified as a plant defence inducer against viruses (Nakashita *et al*., 2003). Wild-type tobacco treated with brassinolide (BL) exhibited enhanced resistance to TMV. BL-treated tobacco plants did not show SA accumulation or induction of PR gene expression, suggesting that BL-induced resistance is distinct from SAR (Nakashita *et al*., 2003). Geminiviruses also interact with the BR signalling pathway. Viral C4 (or AC4 in some viruses) interacts with BRASSINOSTEROID-INSENSITIVE 2 (BIN2), which is a negative regulator of BR signalling (Piroux *et al*., 2007). Although the functional relevance of this interaction remains to be investigated, ectopic expression of the BCTV C4 protein in *A. thaliana* drastically alters plant development, possibly through the disruption of multiple hormonal pathways (Mills-Lujan and Deom, 2010). A BR receptor, the LRR-RLK brassinosteroid insensitive-1 (BRI1), and PRRs interact with the co-receptor BAK1 in a ligand-dependent manner. BAK1 was also found to be essential for plant basal immunity during compatible interactions with RNA viruses. For example, TCV, ORMV and TMV accumulated to higher levels in the *bak1-4* and *bak1-5* mutants than in wild-type plants (Korner *et al*., 2013).

Previous studies showed that the ethylene (ET) pathway might play an important role in antiviral defence (Fischer and Dröge-Laser, 2004; Love *et al*., 2005, 2007). Overexpression of NtERF5, an ET-responsive transcription factor, conferred enhanced resistance to TMV infection, showing reduced size of local HR lesions and impaired systemic spreading of the virus (Fischer and Dröge-Laser, 2004). Mutations in ET signalling were also reported to alter plant susceptibility to viruses. Two arabidopsis ET signalling mutants, *etr1* and *ein2*, showed reduced susceptibility to *Cauliflower mosaic virus* (CaMV) infection (Love *et al*., 2005, 2007). The transcription factor WRKY8, which mediates the ET signalling pathway, is involved in the response against TMV-cg (crucifer-infecting *Tobacco mosaic virus*) (Chen *et al*., 2013). In *wrky8* mutants, several ET-synthesized or responsive transcription factors, such as ACS6 and ERF104, were more strongly induced in TMV-cg systemically infected leaves. Functional analysis using mutants showed that the *acs6*, *erf104* and *ein2* mutants had reduced accumulation of TMV-cg RNA in systemically infected leaves compared with the wild type, indicating an important role for ET in anti-TMV-cg defence. The ET signalling pathway was also correlated with TuMV-initiated suppression of defence responses and enhanced aphid reproduction in plants (Casteel *et al*., 2015). Transgenic expression of Nia-Pro (nuclear inclusion a-protease domain) in arabidopsis alters ethylene responses and suppresses aphid-induced callose formation in an ET-dependent manner.

Abscisic acid (ABA) plays a key role in modulating plant responses to different biotic and abiotic stresses. Although the involvement of ABA in biotic stress has been studied extensively, the roles of ABA in viral replication and movement are not well characterized (Alazem *et al*., 2014, 2015). Previous studies suggested virus-induced changes in ABA metabolism during infection (Whenham *et al*., 1986; Fraser and Whenham, 1989). Tomato plants harbouring the *Tm-1* gene for resistance to TMV contain higher concentrations of ABA than susceptible plants (Fraser and Whenham, 1989). Exogenous applications of ABA reduced the systemic accumulation of TMV-cg. Mutations in ABA deficient 1, ABA deficient 2, ABA deficient 3 or *abi4* accelerated systemic TMV-cg accumulation in arabidopsis (Chen *et al*., 2013). ABA2 has also been shown to play a role in the accumulation of *Bamboo mosaic potexvirus* (BaMV) and CMV (Alazem *et al*., 2014). Mutants downstream of ABA2 (*aao3*, *abi1-1*, a*bi3-1* and *abi4-1*) were susceptible to BaMV. The *aba2-1* mutant showed decreased accumulation of BaMV (+)RNA, (–)RNA and coat protein, with the most dramatic effect being observed for (–)RNA. ABA is also involved in the increase in callose deposition on plasmodesmata by inhibiting β-1,3-glucanase transcription, which may restrict cell to cell movement of the virus and enhance resistance (Beffa *et al*., 1996; Rezzonico *et al*., 1998; Mauch-Mani and Mauch, 2005).

Viral infections may also disturb auxin, cytokinin and gibberellin signalling pathways. The replicase protein of TMV interacts with the related Aux/IAA proteins in arabidopsis and tomato, leading to modifications in auxin-mediated gene regulation and disease development (Padmanabhan *et al*., 2005, 2008). The geminivirus *South African cassava mosaic virus* [ZA:99] activated expression of auxin-inducible genes in arabidopsis (Pierce and Rey, 2013). In a similar manner, the geminivirus AC2/AL2 protein interacts with an ADK in arabidopsis, leading to increased expression of primary cytokinin-responsive genes (Baliji *et al*., 2010). Gibberellic acid may have a defence role against biotrophic or necrotrophic pathogens via modulation of the balance between SA- and JA/ET-mediated signalling pathways (Robert-Seilaniantz *et al*., 2007; Alazem and Lin, 2015). The P2 protein of the *Rice dwarf virus* (RDV) interacts with ent-kaurene oxidase *in vivo*, a key factor in the biosynthesis of gibberellins, leading to a dwarf phenotype in rice, which was rescued after exogenous application of GA_3 (Zhu *et al*., 2005).

It is quite clear that plant hormones play a critical role in many aspects of plant biology, including development and pathogen defence. During viral infection, symptoms and viral accumulation have been correlated with disturbance in phytohormone levels. Despite advances in the knowledge of hormone-mediated antiviral functions, there are still many questions to be answered, including how cross-talk between hormone pathways modulates the host defence response to impair viral infection.

PROTEASOME DEGRADATION

The UPS plays a central role in a wide range of fundamental plant processes, including degradation and functional modification of cellular proteins, and signalling in response to abiotic and biotic stimuli (Sadanandom *et al*., 2012; Luo, 2016). In the context of virus–plant interactions, the UPS is targeted by many viruses to maintain suitable levels of viral proteins and to achieve a successful infection. However, the UPS also acts as a

host defence mechanism to eliminate viral components (Alcaide-Loridan and Jupin, 2012). Several interactions between viral proteins and components of the ubiquitin and ubiquitin-like protein pathways have been reported. The helper component proteases (HcPro) of *Lettuce mosaic virus* (LMV) and PVY were reported to interact directly with different subunits of the 20S proteasome (Jin *et al*., 2007; Dielen *et al*., 2011). The HcPro from *Papaya ringspot virus* (PRSV) interacts with the papaya homologue of arabidopsis PAA (a1 subunit of the 20S proteasome), and inhibition of the proteasome increased the accumulation of PRSV in papaya and accelerated development of symptoms and viral RNA accumulation (Sahana *et al*., 2012). Transgenic tobacco expressing the geminivirus protein βC1 displayed a reduction in polyubiquitination activity, probably due to interaction between βC1 from *Cotton leaf curl Multan virus* (CLCuMV) and the host ubiquitin-conjugating (UBC) enzyme, SlUBC3, leading to disruption of the UPS (Eini *et al*., 2009). Some viruses depend on interactions with the ubiquitin pathway to achieve a successful infection. The geminivirus BSCTV encodes the protein C2, a transcriptional activator, which binds to *S*-adenosylmethionine decarboxylase 1 (SAMDC1) (Zhang *et al*., 2011). This study suggests that BSCTV C2 attenuates the 26S proteasome-mediated degradation of SAMDC1 to establish a hypomethylated environment to facilitate viral accumulation. The *Turnip yellow mosaic virus* (TYMV) RNA-dependent RNA polymerase (66K) is degraded by the UPS in infected cells, which compromises viral infectivity (Camborde *et al*., 2010). The virus, in turn, makes use of a viral deubiquitinating enzyme (DUB) to stabilize RdRp and contribute positively to infection (Chenon *et al*., 2012).

Plant viruses also use UPS processes to promote virulence. The downregulation of *RPM9*, a 26S proteasome subunit, inhibits the systemic spread of TMV and TUMV in *Nicotiana benthamiana* (Jin *et al*., 2006). The viral replication protein (Rep) from geminiviruses binds to host SUMO-conjugating enzyme 1 (SCE1), which is required for viral infection (Castillo *et al*., 2004; Sanchez-Duran *et al*., 2011). Geminiviruses also interfere with the activity of the COP9 signalosome complex through interaction of the viral protein C2 and the host CSN5 protein, compromising many cellular processes regulated by the CUL1-based SCF ubiquitin E3 ligases (Lozano-Duran *et al*, 2011). In the case of the tombusvirus *Tomato bushy stunt virus* (TBSV), a Cdc34p E2 UBC enzyme has been identified to interact with the TBSV p33 replication protein, promoting its ubiquitination (Li *et al*., 2008). Downregulation of *Cdc34p* compromises tombusvirus replicase activity (Li *et al*., 2008).

Geminiviruses alter the cell cycle of infected host cells to create a suitable environment for viral replication (Hanley-Bowdoin *et al*., 2013). The expression of the pathogenicity factor C4 from *Beet severe curly top virus* (BSCTV) affects the cell cycle in arabidopsis, leading to abnormal cell divisions, and induces a host RING finger protein (RKP), which targets cyclin kinase inhibitors for proteasomal degradation. Mutations in RKP reduced the susceptibility to BSCTV and impaired BSCTV replication in plant cells (Lai *et al*., 2009). Some viruses may also induce host protein degradation to defeat the RNA silencing pathway. The polerovirus silencing suppressor P0 from *Beet western yellows virus* (BWYV) targets AGO1 for degradation in a still unknown proteasome-insensitive mechanism (Baumberger *et al*., 2007).

In summary, the UPS involvement in plant defence mechanisms occurs at different levels, from ubiquitin to the 26S proteasome (Dielen *et al*., 2011). Viruses hijack the host UPS to control the quality of their own proteins and to enhance effectiveness. Concomitantly, plants use this pathway as another layer of resistance, mainly targeting viral proteins for degradation. Over the past decades, many reports have revealed a complex network involving host UPS components and viral proteins from several different groups of plant viruses, which suggests that perturbation of the Ub pathway might be a conserved mechanism in virus–host interactions.

CONCLUSIONS

In response to viral infection, plants activate a multilayered defence response, including immune receptor signalling, RNA silencing, hormone-mediated defence pathways and protein degradation. Viruses, however, can subvert the plant's defence signalling by suppressing the host immune system and/or manipulating the host defence signalling network to their own benefit by affecting hormone signalling or the proteasome degradation pathway. Therefore, a better understanding of plant–virus interaction dynamics is crucial if we are to use the plant immune system rationally and more effectively to control viral infections.

In spite of the significant advances in our knowledge of the antiviral immunity in plants made in the last decade, several questions about the dynamics between the virulence strategy of the viruses and the plant immune system remain. For example, we do not know the identities of the virus-derived PAMPs or plant-derived DAMPs that induce antiviral PTI and the viral effectors that suppress it. Furthermore, antiviral PRRs have not been identified. A better understanding of the repertoire of virus effectors (Avr factor) and the NBS-LRR host targets (R proteins) and their mode of action in activating ETI and/or suppressing PTI will help to define the evolutionary pressure acting upon the host and viruses and determine how to deploy the immune system towards a more efficient control of virus infection. We also need to define the NIK1-mediated suppression of translation as a general or virus-specific antiviral strategy in plants. So far, a sustained NIK1 pathway has been shown to be effective against begomoviruses, one of the largest groups of plant DNA viruses, which cannot circumvent the regulatory mechanism of host translation. Although plant RNA viruses have developed a variety of non-canonical mechanisms to translate their RNAs and overcome the regulatory mechanism of host translation, they interact tightly with the host protein synthesis machinery such that host translation initiation factor-encoded genes can function as recessive resistance genes. Furthermore, the translational repression activity of the effector AGO has been recently demonstrated to play a role in the antiviral RNA silencing mechanism. These examples support the argument that hindering the translation of viral mRNA (globally or specifically) is a promising avenue for virus control. Nevertheless, an emerging theme in the plant immunity scenario is that RNA silencing is connected to the other plant defence layers controlling and co-ordinating protein-based innate

immunity and SAR into a more robust defence. Therefore, strategies for integration of different plant defence layers (innate immunity, SAR and RNAi) in a co-ordinated manner are expected to ensure a robust and more durable defence response against plant viruses for crop protection.

ACKNOWLEDGEMENTS

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico [573600/2008-2 and 447578/2014-6 to E.P.B.F.] and the Fundação de Amparo à Pesquisa do Estado de Minas Gerais [CBB-APQ-03175-13 and CBB-APQ-01491-14 to E.P.B.F.]. I.P.C. was supported by a CAPES graduate fellowship.

LITERATURE CITED

Alazem M, Lin KY, Lin NS. 2014. The abscisic acid pathway has multifaceted effects on the accumulation of bamboo mosaic virus. *Molecular Plant-Microbe Interactions* **27**: 177–189.

Alazem M, Lin NS. 2015. Roles of plant hormones in the regulation of host–virus interactions. *Molecular Plant Pathology* **16**: 529–540.

Alcaide-Loridan, C, Jupin, I. 2012. Ubiquitin and plant viruses, let's play together! *Plant Physiology* **160**: 72–82.

Alamillo JM, Saenz P, Garcia JA. 2006. Salicylic acid-mediated and RNA-silencing defence mechanisms cooperate in the restriction of systemic spread of plum pox virus in tobacco. *The Plant Journal* **48**: 217–227.

Andika IB, Maruyama K, Sun L, Kondo H., Tamada, T, Suzuki, N. 2015. Different Dicer-like protein components required for intracellular and systemic antiviral silencing in *Arabidopsis thaliana*. *Plant Signaling and Behavior* **10**: e1039214.

Ascencio-Ibanez JT, Sozzani R, Lee TJ, *et al.* 2008. Global analysis of Arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. *Plant Physiology* **148**: 436–254.

Baebler S, Witek K, Petek M, *et al.* 2014. Salicylic acid is an indispensable component of the Ny-1 resistance gene-mediated response against Potato virus Y infection in potato. *Journal of Experimental Botany* **65**: 1095–1109.

Baliji S, Lacatus, G and Sunter G. 2010. The interaction between geminivirus pathogenicity proteins and adenosine kinase leads to increased expression of primary cytokinin-responsive genes. *Virology* **402**: 238–247.

Bari R, Jones JDG. 2009. Role of plant hormones in plant defence responses. *Plant Molecular Biology* **69**: 473–488.

Baumberger N, Tsai CH, Lie M, Havecker E, Baulcombe DC. 2007. The polerovirus silencing suppressor P0 targets ARGONAUTE proteins for degradation. *Current Biology* **17**: 1609–1614.

Beffa RS, Hofer RM, Thomas M, Meins F Jr. 1996. Decreased susceptibility to virus disease of β-1,3-glucanase-deficient plants generated by antisense transformation. *The Plant Cell* **8**: 1001–1011.

Bendahmane A, Kohm BA, Dedi C, Baulcombe DC. 1995. The coat protein of potato virus X is a strain-specific elicitor of Rx1-mediated virus resistance in potato. *The Plant Journal* **8**: 933–941.

Bendahmane A, Kanyuka K, Baulcombe DC. 1999. The Rx gene from potato controls separate virus resistance and cell death responses. *The Plant Cell* **11**: 781–792.

Bendahmane A, Querci M, Kanyuka K, Baulcombe DC. 2000. Agrobacterium transient expression system as a tool for the isolation of disease resistance genes: application to the Rx2 locus in potato. *The Plant Journal* **21**: 73–81.

Böhm H, Albert I, Fan L, Reinhard A, Nürnberger T. 2014. Immune receptor complexes at the plant cell surface. *Current Opinion in Plant Biology* **20**: 7–54.

Bologna NG, Voinnet O. 2014. The diversity, biogenesis and activities of endogenous silencing small RNAs in Arabidopsis. *Annual Review Plant Biology* **65**: 473–503.

Bowling SA, Guo A, Cao H, Gordon AS, Klessig DF, Dong X. 1994. A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. *The Plant Cell* **6**: 1845–1857.

Brommonschenkel SH, Frary A, Frary A, Tanksley SD. 2000. The broad-spectrum tospovirus resistance gene Sw-5 of tomato is a homolog of the root-knot nematode resistance gene Mi. *Molecular Plant-Microbe Interactions* **13**: 1130–1138.

Brustolini OJB, Machado JPB, Condori-Apfata JA, *et al.* 2015. Sustained NIK-mediated antiviral signalling confers broadspectrum tolerance to begomoviruses in cultivated plants. *Plant Biotechnology Journal* **13**: 1300–1311.

Buchmann RC, Asad S, Wolf JN, Mohannath G, Bisaro DM. 2009. Geminivirus AL2 and L2 proteins suppress transcriptional gene silencing and cause genome-wide reductions in cytosine methylation. *Journal of Virology* **83**: 5005–5013.

Camborde L, Planchais S, Tournier V, *et al.* 2010. The ubiquitin–proteasome system regulates the accumulation of Turnip yellow mosaic virus RNA-dependent RNA polymerase during viral infection. *The Plant Cell* **22**: 3142–3152.

Campos L, Granell P, Tarraga S, *et al.* 2014. Salicylic acid and gentisic acid induce RNA silencing-related genes and plant resistance to RNA pathogens. *Plant Physiology and Biochemistry* **77**: 35–43.

Candresse T, Marais A, Faure C, Dubrana MP, Gombert J, Bendahmane, A. 2010. Multiple coat protein mutations abolish recognition of Pepino mosaic potexvirus (PepMV) by the potato Rx resistance gene in transgenic tomatoes. *Molecular Plant-Microbe Interactions* **23**: 376–383.

Caplan JL, Mamillapalli P, Burch-Smith TM, Czymmek K, Dinesh-Kumar SP. 2008. Chloroplastic protein NRIP1 mediates innate immune receptor recognition of a viral effector. *Cell* **132**: 449–462.

Carbonell, A, Carrington, JC. 2015. Antiviral roles of plant ARGONAUTES. *Current Opinion in Plant Biology* **27**: 111–117.

Carvalho CM, Santos AA, Pires SR, *et al.* 2008. Regulated nuclear trafficking of rpL10A mediated by NIK1 represents a defense strategy of plant cells against virus. *PLoS Pathogens* **4**: e1000247.

Casteel C, De Alwis M, Bak A, Dong H, Steven A, Jander G. 2015. Disruption of ethylene responses by Turnip mosaic virus mediates suppression of plant defense against the aphidvector, Myzus persicae. *Plant Physiology* **169**: 209–218.

Castillo AG, Kong LJ, Hanley-Bowdoin L, Bejarano ER. 2004. Interaction between a geminivirus replication protein and the plant sumoylation system. *Journal of Virology* **78**: 2758–2769.

Chen H, Zhang Z, Teng K, *et al.* 2010. Upregulation of LSB1/GDU3 affects geminivirus infection by activating the salicylic acid pathway. *The Plant Journal* **62**: 12–23.

Chen L, Zhang L, Li D, Wang F, Yu D. 2013. WRKY8 transcription fact functions in the TMV-cg defense response by mediating both abscisic acid a ethylene signaling in Arabidopsis. *Proceedings of the Nationtal Academy of Sciences. USA* **110**: E1963–E1971.

Chenon M, Camborde L, Cheminant S, Jupin I. 2012. A viral deubiquitylating enzyme targets viral RNA-dependent RNA polymerase andaffects viral infectivity. *EMBO Journal* **31**: 741–75.

Chisholm ST, Mahajan SK, Whitham SA, Yamamoto ML, Carrington JC. 2000. Cloning of the Arabidopsis RTM1 gene, which controls restriction of long-distance movement of tobacco etch virus. *Proceedings of the Nationtal Academy of Sciences, USA* **97**: 489–494.

Cosson P, Schurdi-Levraud V, Le QH, *et al.* 2012. The RTM resistance to potyviruses in *Arabidopsis thaliana*: natural variation of the RTM genes and evidence for the implication of additional genes. *PLoS One* **7**: e39169.

Csorba T, Kontra L, Burgyan J. 2015. Viral silencing suppressors: tools forged to fine-tune host–pathogen coexistence. *Virology* **479–480**: 85–103.

Dangl JL, Jones JDG. 2001. Plant pathogens and integrated defence responses to infection. *Nature* **411**: 826–833.

Deleris, A, Gallego-Bartolome, J, Bao J, Kasschau KD, Carrington JC, Voinnet, O. 2006. Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science* **313**: 68–71.

Dielen AS, Sassaki FT, Walter J, Michon T, Menard G. 2011. The 20S proteasome alpha-5 subunit of Arabidopsis thaliana carries an RNase activity and interacts in planta with the lettuce mosaic potyvirus HcPro protein. *Molecular Plant Pathology* **12**: 137–150.

Dodds PN, Rathjen JP. 2010. Plant immunity: towards an integrated view of plant–pathogen interactions. *Nature Review Genetics* **11**: 539–548.

Donaire L, Barajas D, Martinez-Garcia B, Martinez-Priego L, Pagan I, Llave C. 2008. Structural and genetic requirements for the biogenesis of tobacco rattle virus-derived small interfering RNAs. *Journal of Virology* **82**: 5167–5177.

Eini O, Dogra S, Selth LA, Dry IB, *et al.* 2009. Interaction with a host ubiquitin-conjugating enzyme is required for the pathogenicity of a

geminiviral DNA beta satellite. *Molecular Plant Microbe-Interactions* **22**: 737–746.

Farnham G, Baulcombe DC. 2006. Artificial evolution extends the spectrum of viruses that are targeted by a disease-resistance gene from potato. *Proceedings of the National Academy of Sciences, USA* **103**: 18828–18833.

Fischer U, Dröge-Laser W. 2004. Overexpression of NtERF5, a new member of the tobacco ethylene response transcription factor family enhances resistance to tobacco mosaic virus. *Molecular Plant-Microbe Interactions* **17**: 1162–1171.

Fontes EP, Santos AA, Luz DF, Waclawovsky AJ, Chory J. 2004. The geminivirus nuclear shuttle protein is a virulence factor that suppresses transmembrane receptor kinase activity. *Genes and Development* **18**: 2545–2556.

Fraser, RSS, Whenham RJ. 1989. Abscisic-acid metabolism in tomato plant-sinfected with tobacco mosaic-virus – relationships with growth, symptoms and the Tm-1 gene for Tmv resistance. *Physiological and Molecular Plant Pathology* **34**: 215–226.

Gaffney T, Friedrich L, Vernooij B, *et al.* 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* **261**: 754–756.

Garcia-Ruiz H, Takeda A, Chapman EJ, *et al.* 2010. Arabidopsis RNA-dependent RNA polymerases and dicer- like proteins in antiviral defense and small interfering RNA biogenesis during turnip mosaic virus infection. *The Plant Cell* **22**: 481–496.

Gururani MA, Venkatesh J, Upadhyaya CP, Nookaraju A, Pandey SK, Park SW. 2012. Plant disease resistance genes: current status and future directions. *Physiological and Molecular Plant Pathology* **78**: 51–65.

Hanley-Bowdoin L, Bejarano ER, Robertson D, Mansoor S. 2013. Geminiviruses: masters at redirecting and reprogramming plant processes. *Nature Reviews Microbiology* **11**: 777–788.

Hong W, Qian D, Sun R, *et al.* 2015. *OsRDR6* plays role in host defense against double-stranded RNA virus, *Rice Dwarf Phytoreovirus*. *Scientific Reports* **5**: 11324.

Hoser R, Zurczak M, Lichocka M, *et al.* 2013. Nucleocytoplasmic partitioning of tobacco N receptor is modulated by SGT1. *New Phytologist* **200**: 158–171.

Iriti M, Varoni EM. 2014. Chitosan-induced antiviral activity and innate immunity in plants. *Environmental Science and Pollution Research* **22**: 2935–2944.

Ishibashi K, Ishikawa M. 2013. The resistance protein Tm-1 inhibits formation of a Tomato mosaic virus replication protein–host membrane protein complex. *Journal of Virology*, **87**: 7933–7939.

Ivanov KI, Eskelin K, Basic M, *et al.* 2016. Molecular insights into the function of the viral RNA silencing suppressor HCPro. *The Plant Journal* **85**: 30–45.

Incarbone M, Dunoyer P. 2013. RNA silencing and its suppression: novel insights from in planta analyses. *Trends in Plant Science* **18**: 382–392.

Jackson RJ, Hellen CU, Pestova TV. 2010. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nature Reviews Molecular Cell Biology* **11**: 113–127.

Jiang L, Qian D, Zheng H, *et al.* 2012. RNA-dependent RNA polymerase 6 of rice (*Oryza sativa*) plays role in host defense against negative-strand RNA virus, Rice stripe virus. *Virus Research* **163**: 512–519.

Jin H, Li S, Villegas A Jr. 2006. Down-regulation of the 26S proteasome subunit RPN9 inhibits viral systemic transport and alters plant vascular development. *Plant Physiology* **142**: 651–661.

Jin Y, Ma D, Dong J, *et al.* 2007. HC-Pro protein of potato virus Y can interact with three Arabidopsis 20S proteasome subunits in planta. *Journal of Virology* **81**: 12881–12888.

Jones JDG, Dangl JL. 2006. The plant immune system. *Nature* **444**: 323–329.

Jovel J, Walker M, Sanfaçon H. 2011. Salicylic acid-dependent restriction of tomato ringspot virus spread in tobacco is accompanied by a hypersensitive response, local RNA silencing, and moderate systemic resistance. *Molecular Plant-Microbe Interactions* **24**: 706–718.

Kadota Y, Shirasu K. 2012. The HSP90 complex of plants. *Biochim Biophys Acta* **1823**: 689–697.

Kang BC, Yeam I, Jahn MM. 2005. Genetics of plant virus resistance. *Annual Review of Phytopathology* **43**: 581–562.

Kørner CJ, Klauser D, Niehl A, *et al.* 2013. The immunity regulator BAK1 contributes to resistance against diverse RNA viruses. *Molecular Plant-Microbe Interactions* **26**: 1271–1280.

Lai J, Chen H, Teng K, *et al.* 2009. RKP, a RING finger E3 ligase induced by BSCTV C4 protein, affects geminivirus infection by regulation of the plant cell cycle. *The Plant Journal* **57**: 905–917.

Landeo-Rios Y, Navas-Castillo J, Moriones E, Canizare MC. 2015. The p22 RNA silencing suppressor of the crinivirus *Tomato chlorosis virus* preferentially binds long dsRNAs preventing them from cleavage. *Virology* **488**: 129–136.

Li Z, Barajas D, Panavas T, Herbst DA,Nagy PD. 2008. Cdc34p ubiquitin-conjugating enzyme is a component of the tombusvirus replicase complex and ubiquitinates p33 replication protein. *Journal of Virology* **82**: 6911–6926.

Liebrand TWH, van den Burg HA, Joosten MHAJ. 2014. Two for all: receptor-associated kinases SOBIR1 and BAK1. *Trends in Plant Science* **19**: 123–132.

Liu JZ, Horstman, HD, Braun E, *et al.* 2011. Soybean homologs of MPK4 negatively regulate defense responses and positively regulate growth and development. *Plant Physiology* **157**: 1363–1378.

Love AJ, Yun BW, Laval V, Loake GJ, Milner JJ. 2005. Cauliflower mosaic virus, a compatible pathogen of Arabidopsis, engages three distinct defense-signaling pathways and activates rapid systemic generation of reactive oxygen species. *Plant Physiology* **139**: 935–948.

Love AJ, Laval V, Geri C, *et al.* 2007. Components of Arabidopsis defense- and ethylene-signaling pathways regulate susceptibility to Cauliflower mosaic virus by restricting long-distance movement. *Molecular Plant-Microbe Interactions* **20**: 659–670.

Lozano-Duran R, Rosas-Diaz T, Luna AP, Bejarano ER. 2011. Identification of host genes involved in geminivirus infection using a reverse genetics approach. *PLos One* **6**: e22383.

Luo H. 2016. Interplay between the virus and the ubiquitin–proteasome system: molecular mechanism of viral pathogenesis. *Current Opinion in Virology* **17**: 1–10.

Machado JPB, Brustolini OJB, Mendes GC, Santos AA, Fontes EPB. 2015. NIK1, a host factor specialized in antiviral defense or a novel general regulator of plant immunity? *Bioessays* **37**: 1236–1242.

Macho AP, Zipfel C. 2014. Plant PRRs and the activation of innate immune signaling. *Molecular Cell* **54**: 263–272.

Mandadi KK, Scholthof K-BG. 2013. Plant immune responses against viruses: how does a virus cause disease? *The Plant Cell* **25**: 1489–505.

Mann KS, Johnson KN, Carroll BJ, Dietzgen RG. 2016. Cytorhabdovirus P protein suppresses RISC-mediated cleavage and RNA silencing amplification in planta. *Virology* **490**: 27–40.

Mariano AC, Andrade MO, Santos AA, *et al.* 2004. Identification of a novel receptor-like protein kinase that interacts with a geminivirus nuclear shuttle protein. *Virology* **318**: 24–31.

Mauch-Mani B, Mauch F. 2005. The role of abscisic acid in plant–pathogen interactions. *Current Opinion in Plant Biology* **8**: 409–414.

Mills-Lujan K, Deom CM. 2010. Geminivirus C4 protein alters Arabidopsis development. *Protoplasma* **239**: 95–110.

Nakashita H, Yasuda M, Nitta T, *et al.* 2003. Brassinosteroid functions in a broad range of disease resistance in tobacco and rice. *The Plant Journal* **33**: 887–898.

Nicaise V. 2014. Crop immunity against viruses: outcomes and future challenges. *Frontiers in Plant Science* **5**: 660.

Nolen B, Taylor S, Ghosh G. 2004. Regulation of protein kinases: controlling activity through activation segment conformation. *Molecular Cell* **15**: 661–675.

Oka K, Kobayashi M, Mitsuhara I, Seo S. 2013. Jasmonic acid negatively regulates resistance to Tobacco mosaic virus in tobacco. *Plant and Cell Physiology* **54**: 1999–2010.

Padmanabhan MS, Goregaoker SP, Golem S, Shiferaw H, Culver, JN. 2005. Interaction of the tobacco mosaic virus replicase protein with the Aux/IAA protein PAP1/IAA26 is associated with disease development. *Journal of Virology* **79**: 2549–2558.

Padmanabhan MS, Kramer SR, Wang X, Culver JN. 2008. Tobacco mosaic virus replicase–auxin/indole acetic acid protein interactions: reprogramming the auxin response pathway to enhance virus infection. *Journal of Virology* **82**: 2477–2485.

Parent JS, Bouteiller N, Elmayan T, Vaucheret H. 2015. Respective contributions of Arabidopsis DCL2 and DCL4 to RNA silencing. *The Plant Journal* **81**: 223–232.

Pierce EJ, Rey ME. 2013. Assessing global transcriptome changes in response to South African cassava mosaic virus [ZA99] infection in susceptible *Arabidopsis thaliana*. *PLos One* **8**: e67534.

Pieterse CMJ, van der Does D, Zamioudis C, Leon-Reyes A, van Wees SCM. 2012. Hormonal modulation of plant immunity. *Annual Reviews of Cell and Developmental Biology* **28**: 489–521.

Piroux N, Saunders K, Page A, Stanley J. 2007. Geminivirus pathogenicity protein C4 interacts with *Arabidopsis thaliana* shaggy-related protein kinase AtSKη, a component of the brassinosteroid signalling pathway. *Virology* **362**: 428–440.

Postma J, Liebrand TW, Bi G, *et al.* 2016. Avr4 promotes Cf-4 receptor-like protein association with the BAK1/SERK3 receptor-like kinase to initiate receptor endocytosis and plant immunity. *New Phytologist* **210**: 627–642.

Qu F, Ye X, Morris,TJ. 2008. *Arabidopsis* DRB4, AGO1, AGO7, and RDR6 participate in a DCL4-initiated antiviralRNA silencing pathway negatively regulated by DCL1. *Proceedings of the Nationtal Academy of Sciences, USA* **105**: 14732–14737.

Rairdan GJ, Collier SM, Sacco MA, Baldwin TT, Boettrich T, Moffett P. 2008. The coiled-coil and nucleotide binding domains of the Potato Rx disease resistance protein function in pathogen recognition and signaling. *The Plant Cell* **20**: 739–751.

Raja P, Sanville BC, Buchmann RC, Bisaro DM. 2008. Viral genome methylation as an epigenetic defense against geminiviruses. *Journal of Virology* **82**: 8997–9007.

Raja P, Jackel JN, Li S, Heard IM, Bisaro DM. 2014. Arabidopsis double-stranded RNA binding protein DRB3 participates in methylation-mediated defense against geminiviruses. *Journal of Virology* **88**: 2611–2622.

Rezzonico E, Flury N, Meins F Jr, Beffa R. 1998. Transcriptional down-regulation by abscisic acid of pathogenesis-related beta-1,3-glucanase genes in tobacco cell cultures. *Plant Physiology* **117**: 585–592.

Revers F, Nicaise V. 2014. Plant resistance to infection by viruses. *eLS* doi: 10.1002/9780470015902.a0000757.pub3.

Robert-Seilaniantz A, Navarro L, Bari R, Jones JD. 2007. Pathological hormone imbalances. *Current Opinion in Plant Biology* **10**: 372–379.

Rocha CS, Santos AA, Machado JPB, Fontes EPB. 2008. The ribosomal protein L10/QM-like protein is a component of the NIK mediated antiviral signaling. *Virology* **380**: 165–169.

Rodriguez-Negrete E, Lozano-Durán R, Piedra-Aguilera A, Cruzado L, Bejarano ER, Castillo AG. 2013. Geminivirus Rep protein interferes with the plant DNA methylation machinery and suppresses transcriptional gene silencing. *New Phytologyst* **199**: 464–475.

Ruiz-Ferrer V, Voinnet O. 2009. Roles of plant small RNAs in biotic stress responses. *Annual Review of Plant Biology* **60**: 485–510.

Sadanandom A, Bailey M, Ewan R, Lee J, Nelis S. 2012. The ubiquitin–proteasome system: central modifier of plant signalling. *New Phytologist* **196**: 13–28.

Sahana N, Kaur H, Basavaraj, *et al.* 2012. Inhibition of the host proteasome facilitates papaya ringspot virus accumulation and proteosomal catalytic activity is modulated by viral factor HcPro. *PLoS One* **7**: e52546.

Sakamoto T, Deguchi M, Brustolini OJB, *et al.* 2012. The tomato RLK superfamily: phylogeny and functional predictions about the role of the LRRII-RLK subfamily in antiviral defense. *BMC Plant Biology* **12**: 229.

Sánchez-Durán MA, Dallas MB, Ascencio-Ibañez JT, *et al.* 2011. Interaction between geminivirus replication protein and the SUMO-conjugating enzyme is required for viral infection. *Journal of Virology* **85**: 9789–9800.

Santos AA, Carvalho CM, Florentino LH, *et al.* 2009. Conserved threonine residues within the A-loop of the receptor NIK differentially regulate the kinase function required for antiviral signaling. *PLoS One* **4**: e5781.

Santos AA, Lopes KVG, Apfata JAC, Fontes EPB. 2010. NSP interacting kinase, NIK: a transducer of plant defence signalling. *Journal of Experimental Botany* **61**: 3839–3845.

Soitamo AJ, Jada B, Lehto K. 2012. Expression of geminiviral AC2 RNA silencing suppressor changes sugar and jasmonate responsive gene expression in transgenic tobacco plants. *BMC Plant Biology* **12**: 204.

Szittya G, Burgyan J. 2013. RNA interference-mediated intrinsic antiviral immunity in plants. *Current Topics in Microbiology and Immunology* **371**: 153–181.

Takahashi H, Suzuki M, Natsuaki K, Shigyo T, Hino K, *et al.* 2001. Mapping the virus and host genes involved in the resistance response in cucumber mosaic virus infected Arabidopsis thaliana. *Plant and Cell Physiology* **42**: 340–347.

Takahashi H, Kanayama Y, Zheng MS, *et al.* 2004. Antagonistic interactions between the SA and JA signaling pathways in Arabidopsis modulate expression of defense genes and gene-for-gene resistance to cucumber mosaic virus. *Plant and Cell Physiology* **45**: 803–809.

Tameling WI, Nooijen C, Ludwig N, *et al.* 2010. RanGAP2 mediates nucleocytoplasmic partitioning of the NB-LRR immune receptor Rx in the Solanaceae, thereby dictating Rx function. *The Plant Cell* **22**: 4176–4194.

Ueda H, Yamaguchi Y, Sano H. 2006. Direct interaction between the tobacco mosaic virus helicase domain and the ATP-bound resistance protein, N factor during the hypersensitive response in tobacco plants. *Plant Molecular Biology* **61**: 31–45.

Vallejos CE, Astua-Monge G, Jones V, *et al.* 2006. Genetic and molecular characterization of the I locus of Phaseolus vulgaris. *Genetics* **172**: 1229–1242.

Vlot AC, Dempsey DA, Klessig DF. 2009. Salicylic acid, a multifaceted hormone to combat disease. *Annual Review Phytopathology* **47**: 177–206.

Wang H, Hao L,Shung CY, Sunter G,Bisaro DM. 2003. Adenosine kinase is inactivated by geminivirus AL2 and L2 proteins. *The Plant Cell* **15**: 3020–3032.

Wang H, Buckley KJ, Yang X, Buchmann RC, Bisaro DM. 2005. Adenosine kinase inhibition and suppression of RNA silencing by geminivirus AL2 and L2 proteins. *Journal of Virology* **79**: 7410–7418.

Wang XB, Wu Q, Ito T, *et al.* 2010. RNAi-mediated viral immunity requires amplification of virus-derived siRNAs in *Arabidopsis thaliana*. *Proceedings of the Nationtal Academy of Sciences, USA* **107**: 484–489.

Whenham RJ, Fraser RSS, Brown, LP, Payne JA. 1986. Tobacco Mosaic Virus-induced increase in abscisic-acid concentration in tobacco leaves – intracellular location in light and dark-green areas, and relationship to symptom development. *Planta* **168**: 592–598.

Witham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B. 1994. The product of the tobacco mosaic virus resistance gene N: similarity to toll and the interleukin-1 receptor. *Cell* **78**: 1101–1115.

Whitham SA, Anderberg RJ, Chisholm ST, Carrington JC. 2000. Arabidopsis RTM2 gene is necessary for specific restriction of tobacco etch virus and encodes an unusual small heat shock-like protein. *The Plant Cell* **12**: 569–582.

Whitham SA, Yang C, Goodin MM. 2006. Global impact: elucidating plant responses to viral infection. *Molecular Plant-Microbe Interaction* **19**: 1207–1215.

Wieczorek P, Obrepalska-Steplowska A. 2015. Suppress to survive – implication of plant viruses in PTGS. *Plant Molecular Biology Report* **33**: 335–346.

Yang H, Gou X, He K, Xi D, *et al.* 2010. BAK1 and BKK1 in *Arabidopsis thaliana* confer reduced susceptibility to turnip crinkle virus. *European Journal of Plant Pathology* **127**: 149–156.

Yang JY, Iwasaki M, Machida C, Machida Y, Zhou X, Chua N. 2008. βC1, the pathogenicity factor of TYLCCNV, interacts with AS1 to alter leaf development and suppress selective jasmonic acid responses. *Genes and Development* **22**: 2564–2577.

Yang X, Xie Y, Raja P, *et al.* 2011. Suppression of methylation-mediated transcriptional gene silencing by betaC1–SAHH protein interaction during geminivirus-betasatellite infection. *PLoS Pathogens* **7**: e1002329.

Zhang C, Wu Z, Li Y, Wu J.2015. Biogenesis, function, and applications of virus-derived small RNAs in plants. *Frontiers in Microbiology* **6**: 1237.

Zhang X, Zhang X, Singh J, Li D, Qu F. 2012. Temperature-dependent survival of turnip crinkle virus-infected Arabidopsis plants relies on an RNA silencing-based defense that requires dcl2,AGO2,and HEN1. *Journal of Virology* **86**: 6847–6854.

Zhang Z, Chen H, Huang X, *et al.* 2011. BSCTV C2 attenuates the degradation of SAMDC1 to suppress DNA methylation-mediated gene silencing in Arabidopsis. *The Plant Cell* **23**: 273–288.

Zhu SF, Gao F, Cao XS, *et al.* 2005. The rice dwarf virus P2 protein interacts with ent-kaurene oxidases *in vivo*, leading to reduced biosynthesis of gibberellins and rice dwarf symptoms. *Plant Physiology* **139**: 1935–1945.

Zhu S, Jeong RD, Lim GH, *et al.* 2013. Double-stranded RNA-binding protein 4 is required for resistance signaling against viral and bacterial pathogens. *Cell Reports* **4**: 1168–1184.

Zipfel C. 2014. Plant pattern-recognition receptors. *Trends in Immunology* **35**: 345–351.

Zorzatto C, Machado JPB, Lopes KVG, *et al.* 2015. NIK1-mediated translation suppression functions as a plant antiviral immunity mechanism. *Nature* **520**: 679–682.

Chapter II

ATWWP1, A WW DOMAIN-CONTAINING PROTEIN, FORMS NUCLEAR BODIES THAT COUNTERACT BEGOMOVIRUS INFECTION

ATWWP1, A WW DOMAIN-CONTAINING PROTEIN, FORMS NUCLEAR BODIES THAT COUNTERACT BEGOMOVIRUS INFECTION

Abstract

As DNA viruses, which replicate in the nucleus of infected cells, the bipartite begomoviruses (*Geminiviridae* family) encode the nuclear shuttle protein (NSP) to facilitate the translocation of viral DNA from the nucleus to the cytoplasm via nuclear pores. This intracellular trafficking of NSP-DNA complexes is accessorized by the NSP-interacting GTPase (NIG) at the cytosolic side. Here, we report the characterization of AtWWP1, a WW domain-containing protein, identified as a NIG partner. We demonstrated that AtWWP1 forms nuclear bodies (NBs) via its WW domains and relocates NIG from the cytoplasm to the nucleus where it is confined to AtWWP1-NBs. Therefore, the NIG-AtWWP1 interaction, which also occurs via the WW domains, may interfere with the NIG pro-viral function that is associated with its cytosolic localization. Consistent with this hypothesis, loss of *AtWWP1* function debilitates further the plant upon begomovirus infection and overexpression of *AtWWP1* confers tolerance to begomovirus. The antiviral function of AtWWP1-NBs, however, may be antagonized by viral infection, which was demonstrated to induce either a decrease in the number or disruption of AtWWP1-NBs. Collectively, our data establish that AtWWP1 organizes nuclear structures as nuclear foci, which provide intrinsic immunity against begomovirus infection.

Introduction

Among the environmental limitations that affect plant growth, viruses cause major crop losses worldwide as they often suppress the plant defenses and severely limit the productivity of relevant crops. Consequently, they represent a serious threat to global food security and the mechanisms by which they establish themselves and propagate within plant cells are the focus of intensive studies. As obligatory intracellular parasites, plant viruses interact profoundly with the host cell machinery for the completion of their life cycle. These interactions initiate with the virus invasion of host cells and persist during viral genome replication and expression, cell-to-cell and long distance movement of virus particles until their insect transmission to another host (Nagy, 2016; Hanley-Bowdoin *et al*., 2013). Therefore, the functional components of the host cell machinery are excellent targets of viral proteins, as the viruses have to usurp the host basic functions to establish a productive infection. Accordingly, eukaryotic viruses, in general, have been extensively used as templates to uncover the basic cellular functions of host cells (Hanley-Bowdoin *et al*., 2013). A major example of plant viruses as a model system is the *Geminiviridae* family, which comprises viruses with circular single-stranded DNA genome of ~2.9-5.2 kb, encapsidated within a twinned quasi-icosahedral virus particle (Hanley-Bowdoin *et al*., 2013).

Begomovirus represents the largest genus of the *Geminiviridae* family, which consists of whitefly-transmitted viruses with either monopartite or bipartite genomes that inflict severely a wide range of relevant crops in tropical and subtropical regions (Rojas *et al.,* 2005; Inoue-Nagata *et al*., 2016). Current climate changes are expected to alter the whitefly distribution across the globe, posing an additional threat to agriculture worldwide. The genome of the bipartite begomoviruses is split between

two genomic components, designated DNA-A and DNA-B (Fondong, 2013; Hanley-Bowdoin *et al*., 2013). The DNA-A component encodes five proteins, which are involved in DNA replication (replication protein, Rep, and replication enhancer protein, REn), transcription of viral genes (transactivation protein, TrAP), suppression of plant defenses (Trap and AC4) and encapsidation of viral DNA (coat protein, CP). DNA-B encodes two movement proteins, the nuclear shuttle protein, NSP (BV1), and the movement protein, MP (BC1), both required for systemic infection. As the ssDNA begomoviruses replicate via double-stranded DNA intermediates in the nuclei of infected cells, they require two movement proteins to move the newly replicated DNA from the nucleus to the cytosol via nuclear pores and then to adjacent cells via plasmodesma. In addition to functioning as a suppressor of the immune receptor NSP-interacting kinase 1 (NIK1; Calil and Fontes, 2016; Gouveia *et al*., 2016; Machado *et al*., 2017), NSP facilitates the nucleocytoplasmic movement of the newly synthesized viral DNA and acts in concert with MP to move the viral DNA to adjacent, uninfected cells (Gafni and Epel, 2002; Lazarowitz and Beachy, 1999; Rojas *et al*., 2005).

The mechanistic model for begomoviral DNA intracellular trafficking preconizes that NSP binds to newly-replicated viral DNA in the nuclei of infected cells and utilizes the nuclear export machinery to move the viral DNA to the cytoplasm (Lazarowitz and Beachy, 1999; Ward and Lazarowitz, 1999; Gafni and Epel, 2002). At the cytoplasmic side of the nuclear pore complex, the NSP-interacting GTPase (NIG) binds to NSP to facilitate the intracellular transport of viral DNA-NSP complexes from the nuclear envelope to the cortical cytoplasm where it is replaced by MP (Carvalho *et al*., 2008a; 2008b). Evidence for NIG function in the viral DNA-NSP trafficking relies on the demonstration that NIG interacts *in vivo* and *in vitro* with

NSP, is capable of moving NSP-DNA complexes from the nucleus to the cytoplasm and functions as a pro-viral factor during begomovirus infection (Carvalho *et al*., 2008a; 2008b). Furthermore, the interaction between NSP and NIG resembles the complex formed between the nuclear shuttle protein Rev from human immunodeficiency virus type 1 (HIV-1) and human Rev-Interacting Protein (hRIP), which has been shown to be involved in the mechanism for the release of viral RNAs from the nuclear periphery to the cytoplasm (Sánchez-Velar *et al*., 2004). In addition to displaying similar function in virus infection, NIG and hRIP share structural features, including an ArfGap domain and a B-box zinc finger motif, but they differ in several other relevant aspects (Pollard *et al*., 1998; Carvalho *et al*., 2008a; 2008b). These differences include a GTPase activity displayed by NIG but not by hRIP, and the subcellular localization of the homolog proteins. While hRIP is nuclear localized, NIG is distributed unevenly in the cytosol and concentrates around the nuclear envelope. Although the NIG localization is consistent with a role in assisting the release of exported proteins from the nuclear pore complexes at the cytoplasmic side, frequently, the nucleocytoplasmic transport proteins shuttle between the nucleus and the cytoplasm to dynamically facilitate the intracellular movement of nuclear proteins rather than just tether proteins in subcellular compartments (Wiermer *et al*., 2007). However, attempts to demonstrate a regulated nuclear localization of NIG in response to different stimuli have failed and the function of the protein remains elusive. Because there is no advantage for plant cells to evolve a facilitating transport activity for virus intracellular movement, it is reasonable to predict that NIG acts at cellular protein complexes as well.

To determine the cellular function of NIG, we searched for NIG targets using the yeast two-hybrid system. We show here that a WW domain-containing protein,

designated AtWWP1, forms nuclear bodies (NB) and relocates NIG to the nucleus where it is confined in the AtWWP1-NB. The AtWWP1-mediated subcellular redistribution of NIG is associated with an antiviral immune function, because manipulation of AtWWP1 levels correlates with resistance to begomovirus infection, and AtWWP1-NBs are modified by virus infection and interact with the conserved CSN5A immune hub (Mukhtar *et al*., 2011) via AtWWP1-CSN5A complex formation.

Results

A WW domain-containing protein, designated AtWWP1, interacts with NIG in the nucleus

NIG is a cytosolic GTPase, which also accumulates around the nuclear membrane, and displays a positive role during begomovirus infection (Carvalho *et al*., 2008a; 2008b). Furthermore, NIG exhibits biochemical proprieties, consistent with an involvement in nucleocytoplasmic transport of molecules (Carvalho *et al*., 2008a). In order to gain insights into the cellular function of NIG, we searched for proteins that interact with NIG using yeast two-hybrid screens with a cDNA library from *Arabidopsis thaliana* aerial tissues (Florentino *et al*., 2006) and identified several NIG-interacting proteins. Among the candidates, a partial cDNA from a WW domain-containing protein encoded by the AT2G41020 *locus* was isolated and the encoded protein named AtWWP1.

The interaction between a full-length AtWWP1 and the Pro-rich domain of NIG (PRNIG; that interact with the begomovirus NSP) was first confirmed through the yeast two-hybrid system (Fig. 1A and B). Co-expression of full-length AtWWP1 ORF

fused to the binding domain (BD) PRNIG fused to the activation domain (AD) of GAL4 or vice-versa promoted histidine prototrophy on medium lacking histidine and supplemented with 3AT (3-Amino-1,2,4-triazole; Fig. 1A). The interactions were also confirmed by determining β-galactosidase activity in yeast protein extracts, which was significantly higher in protein extracts from yeast cells co-expressing the chimeric proteins (Fig. 1B). Neither the *HIS3* marker gene nor *lacZ* was activated in yeast cells co-transformed with the controls.

We next examined whether AtWWP1 associated with NIG in *planta* by co-immunoprecipitation assays (CoIP). Full-length AtWWP1-GFP and NIG-6HA were transiently expressed - alone or co-expressed - in *Nicotiana benthamiana* leaves. Anti-GFP antibodies-coupled magnetic beads were used to immunoprecipitate the AtWWP1-NIG complex from whole-cell protein extracts of transfected cells. The presence of NIG-HA was analyzed by western blot and detected using an HA-antibody. The CoIP results indicated that AtWWP1-GFP interacted with NIG-6HA *in vivo*, as (Fig. 1C). The *in vivo* interaction between AtWWP1 and NIG was further confirmed using the bimolecular fluorescence complementation (BiFC) assay (Fig. 1D). *N. benthamiana* leaves were co-infiltrated with *Agrobacterium tumefaciens* cells carrying AtWWP1 and NIG fused to non-fluorescent fragments (N-terminus and C-terminus) from fluorescent yellow protein (YFP). The infiltrated leaves were analyzed by confocal microscopy 3 days after infiltration. YFP reconstituted fluorescence was observed in cells co-infiltrated with nYFP-WWP1 and cYFP-NIG constructs or *vice versa*. In contrast, expression of AtWWP1 or NIG constructs alone fused to nYFP or cYFP did not restore the YFP fluorescence. Although NIG is a cytosolic protein, the interaction between AtWWP1 and NIG was observed in the nucleus of transfected cells. This data supports a putative role of NIG as a nucleocytoplasmic shuttling

protein. Additionally, these results may indicate a possible nuclear function of the NIG-AtWWP1 complex.

AtWWP1 is a 463 aa protein (theoretical Mw: 49814.67; pI: 6.47), which contains two WW domains. The first one comprises the amino acid residues 194-224, and the second one is located at 241-269 amino acid positions (http://pfam.xfam.org/). WW domains are small protein modules, which mediate protein-protein interactions through recognition of proline rich peptide motifs (PRM) and phosphorylated serine/threonine-proline sites (Macias *et al*., 2000; Igham *et al*., 2005; Salah *et al*., 2012). The capacity of this simple domain in interacting with a diverse number of proline-containing ligands imply in a wide functional diversity. In fact, WW domains are present in many different structural and signaling proteins, which are involved in a variety of cellular processes, including RNA transcription and processing, signal transduction, assembly of multiprotein complexes, protein trafficking and stability and control of the cytoskeleton (Salah *et al*., 2012). Although WW domain-containing proteins are largely documented in mammalian organisms, the characterization of plant proteins encoding WW domains is still limited.

To access the diversity of WW-domain containing proteins in plants and to examine the evolutionary relationship of AtWWP1 with other plant proteins harboring the WW domain, we performed a phylogenetic analysis of WW domain-containing proteins from Arabidopsis and relevant agronomic crops (soybean, tomato and rice) using the Bayesian method (Fig. S1A and S1B). The amino acid sequences were recovered from TAIR (http://arabidopsis.org/) and Phytozome v11 databases. The WW domain was predicted with HMMER software using the pfam v.30 database. AtWWP1 is most closely related to uncharacterized proteins from *Glycine Max (*Glyma02G162000; 57.05% identity), *Oriza sativa* (LOC_Os01G68760, 51.33%

identity) and *Solanum lycopersicum* (Solyc11G005110, 53.35% identity; Fig. S1A). In addition to the presence of two WW domains, these proteins also share a conserved C-terminus region. These data suggest that AtWWP1 may play additional roles beyond those involving its WW domain. In Arabidopsis, there are 13 encoded WW domain-containing proteins, most of them are functionally uncharacterized (Fig. S1B). AtWWP1 is phylogenetically grouped with AT3G19840 (20.34% identity), AT1G44910 (22.67% identity) and AT3G19670 (23.44% identity) proteins. This set of proteins is collectively referred to as AtPrp40s (pre-mRNA processing protein 40), named AtPRP40C, AtPRP40A and AtPRP40B, respectively. In yeast, Prp40 is a splicing factor, which associates with U1 small nuclear ribonucleoprotein (snRNP) and also binds to phosphorylated carboxyl-terminal domain (CTD) of the largest subunit of RNA Polymerase II via multiple WW and FF domains (Kao and Siliciano, 1996; Morris and Greenleaf, 2000; Becerra *et al*., 2016). Multiple sequence alignment of AtWWP1 and Arabidopsis Prp40s shows that the WW domains represent the most conserved region (Fig. S1C). In contrast, AtPRP40C lacks the second W residue from the first WW domain. The Arabidopsis Prp40 homologs (AtPRP40A, AtPRP40B and AtPRP40C) also associate with RNA Polymerase II CTD domain (Kang *et al*., 2009). The phylogenetic relationship between AtWWP1 and AtPRP40s suggests that AtWWP1 may function in pre-mRNA processing and transcription.

The human polyglutamine tract-binding protein 1 (PQBP1) is an extensively studied WW domain-containing protein, which is associated with neurological disorders (Waragai *et al*., 1999; Kunde *et al*., 2011; Wang *et al*., 2013). It is predominantly localized in the cell nucleus, where it forms subnuclear inclusion bodies (Okasawa *et al*., 2001; Kunde *et al*., 2011). PQBP1 is functionally related to

pre-mRNA splicing and transcription and, more recently, it was reported to be involved in innate immune signaling (Waragai *et al*., 1999; Okazawa *et al*., 2002; Waragai *et al*., 2002; Wang *et al*., 2013; Yoh *et al*., 2015; Mizuguchi *et al*., 2016). Sequence alignment of AtWWP1 and PQBP1 reveals a conserved region comprising the first WW domain of AtWWP1 and a highly conserved C terminus motif (Fig. S1D). Collectively, these data indicate that AtWWP1 may share functional homology with PQBP1. The evolutionary conservation of the WW domain-containing proteins PQBP1 and AtWWP1 from such diverse species indicates that both proteins may execute basic and related functions in cells.

AtWWP1 localizes in nuclear bodies

The sequence similarity between AtWWP1 and the human PQBP1 raised up the hypothesis that these proteins could also share functional homology and biochemical proprieties. PQBP1 is mainly localized in the nucleus although it is also present in the cytoplasm. In the nucleus, PQBP1 forms intranuclear inclusion bodies, but also displays a diffuse nucleoplasmic distribution (Okasawa *et al*., 2001; Nicolaescu *et al*., 2008). To determine the subcellular localization of AtWWP1, we transiently expressed AtWWP1, under the control of either the native or constitutive promoters, in *N. benthamiana* leaves and monitored the localization of AtWWP1-GFP or AtWWP1-YFP fusion protein by confocal microscopy (Fig. 2A and 2B). We also examined the subcelluar localization of AtWWP1 in Arabidospsis transgenic lines (Fig. 2C). In both systems, AtWWP1 was exclusively localized in the nucleus and displayed both diffused and nuclear body patterns, although to different extents (Fig. 2). AtWWP1 predominantly formed nuclear bodies, as they were present in 89.5% ±

0.07 of transfected cells. In both transiently and stably expressing experiments, the number and size of AtWWP1 nuclear bodies varied broadly, ranging from a few to dozen bodies. The formation of nuclear bodies by expressing AtWWP1 under the control of both native (Fig. 2B) and constitutive (Fig. 2A) promoters indicates that they might represent functional structures rather than merely protein aggregates as a result of its overexpression. These data indicate that AtWWP1 is a nuclear protein, mainly localized in nuclear bodies.

AtWWP1 relocates NIG from the cytoplasm to nuclear bodies

We have shown that AtWWP1 interacts with NIG in the nucleus, although NIG is localized in the cytoplasm in both transient and stable expression assays (Fig. 3A and 3B; Carvalho *et al*., 2008a). In transient expression assays, NIG retains its cytosolic pattern of distribution (in the cytoplasm and around the nuclear envelope) no matter if the tag is fused to the carboxyl-terminus (NIG-mCherry) or amino-terminus (YFP-NIG). In contrast, AtWWP1 is nuclear localized, predominantly confined within NBs. These observations prompted us to investigate whether AtWWP1 could affect NIG function by altering its subcellular localization. To this purpose, we expressed a full-length AtWWP1-GFP and NIG-mCherry in epidermal cells of *N. benthamiana* leaves and analyzed the subcellular localization of both proteins by confocal microscopy. When expressed alone, NIG-mCherry preserved its cytsololic localization pattern, diffused in the cytoplasm and concentrated around the nuclear envelope (Fig. 3A). NIG remained cytosolic localized by changing the tag (YFP) to the N-terminus. However, when co-expressed with AtWWP1, NIG was relocated to the nucleus where it co-localized with AtWWP1 in nuclear bodies (Fig.

3C). Arabidopsis transgenic lines co-expressing AtWWP1-GFP and NIG-mCerry also exhibited subnuclear colocalization, although to a less extent than transiently co-expressing cells (Fig 3D). This discrepancy in colocalization efficiency could be due to differences between the transient and stable-transgenic system. In fact, the accumulation of transiently expressed NIG transcript and protein in *N. benthamiana* leaves is several-fold higher than in Arabidopsis transgenic lines. We postulated that NIG has a high turnover rate *in vivo*. Furthermore, in nature, AtWWP1 is not expressed in roots, as the AtWWP1 promoter displays tissue-specific expression with no activity in roots; thereby, root cells may not have evolved a complete biochemical apparatus to address properly the AtWWP1 biological function (Fig. S2). Collectively, our data show that AtWWP1 and NIG interact directly in the nucleus of plant cells and, when co-expressed, NIG is recruited to AtWWP1 nuclear bodies.

AtWWP1 exhibits antiviral activity against begomovirus infection

NIG was initially identified as a positive contributor to geminivirus infection. *NIG*-overexpressing Arabidopsis plants displayed enhanced susceptibility to geminivirus infection (Carvalho *et al*., 2008a). According to the current hypothesis, NIG interacts with the begomovirus NSP and facilitates the translocation of the viral protein from the nucleus to the cytoplasm, thereby enabling MP–NSP interaction in the cortical cytoplasm (Carvalho *et al*., 2008a; 2008b). As the NIG function in the geminivirus infection is apparently associated with its cellular localization and AtWWP1 has been shown to sequester NIG in the nucleus, we investigated whether AtWWP1 could affect virus infection. The function of AtWWP1 in begomovirus infection was assayed with several different approaches. We first prepared

independent *AtWWP1*-overexpressing transgenic lines, expressing different levels of *AtWWP1* transgene and protein (Fig. S3A and S3B). We also identified a transfer DNA (T-DNA) insertion mutant (*atwwp1*) in the *AtWWP1* gene (Fig S3A and S3C). Then, we determined the *AtWWP1*-induced global variation in gene expression by comparing the transcriptome of AtWWP-GFP-4, *atwwp1* knockout and wild type leaves. Unexpectedly, either overexpression or inactivation of the *AtWWP1* gene promoted a general downregulation of genes involved in immune response (Fig. S4). These results indicate that AtWWP1 may function as an indicator of NB activity, which may regulate transcription of immune genes. This hypothesis predicts that the basal levels of AtWWP1 are tightly controlled; preventing that low or high level of expression would affect the AtWWP1 NB activity. Consistent with this interpretation, the endogenous *AtWWP1* promoter drives very low levels of *AtWWP1* expression in transient assays.

To directly examine whether AtWWP1 is involved in begomovirus infection, the overexpressing lines AtWWP1-1 and AtWWP1-4, the *atwwp1* mutant line and wilt-type plants were inoculated with tandemly repeated CaLCuV DNA-A and DNA-B by biolistic delivery. The course of infection was monitored as previously described (Florentino *et al*., 2006), and viral DNA was detected by PCR. Plants were evaluated according to symptoms of infection and the accumulation of viral DNA, detected by qPCR (Fig. 4). Virus-inoculated wild-type plants displayed typical symptoms of CaLCuV infection, whereas *atwwp1* mutants showed more severe disease symptoms, including extreme growth stunting and intense leaf distortion (Fig. 4A), an accelerated progress of infection compared to wild type (Fig 4B) and a tendency to accumulate a higher level of viral DNA than WT and *AtWWP1*-overexpressing lines (Fig 4C). Therefore, loss of *AtWWP1* function further debilitated the plants upon

begomovirus infection. In contrast, the *AtWWP1*-overexpressing line AtWWP1-4, exhibited attenuated symptoms followed by a delayed course of infection and lower accumulation of viral DNA in the systemically infected leaves as compared to others genotypes (Fig. 4B and 4C). The observed disease symptoms contrasted between the two Arabidopsis *AtWWP1*-overexpressing lines. While AtWWP1_4 plants showed an enhanced tolerant phenotype and reduced accumulation of viral DNA in systemic leaves, AtWWP1_1 plants displayed an infected phenotype and viral DNA accumulation similar to wild type infected plants. These data may be explained by the difference in the levels of *AtWWP1* transcript and protein accumulation in transgenic lines, which is higher in the transgenic AtWWP1_4 comparing to AtWWP1_1 (Fig S3A and S3B). RNA-seq data confirmed that the *AtWWP1* transcript levels in AtWWP-1 lines are only slightly higher than in wild-type leaves (Table S1). The weak susceptibility phenotype displayed by the knockout lines could be explained by functional redundancy as AtWWP1 has three putative homologs in the Arabidopsis genome (Fig. S1B). Our data demonstrated a perfect correlation between transgene expression and AtWWP1 immune function and substantiated the notion that AtWWP1 confers enhanced tolerance to begomovirus.

Begomovirus infection interferes in the organization of AtWWP1 nuclear bodies

As NIG has been shown to potentiate virus infection and confer recessive resistance (Carvalho *et al.,* 2008a), we rationalized that the NIG partner AtWWP1 could enhance begomovirus tolerance by perturbing NIG function. In light of this view, we asked whether the AtWWP1-mediated shift in NIG subcellular distribution would account for the AtWWP1 antiviral activity or whether the immune property of

AtWWP1 would be linked with its NB-forming activity. To address this issue, we examined first whether begomovirus infection would disturb the organization of AtWWP1-NB. Then, we designed a series of experiments to construct first an AtWWP1 mutant defective in nuclear body formation and then we assayed for antiviral activity of the loss-of function mutant upon begomovirus infection in complementation assays.

To assay for begomovirus interference in AtWWP1-NB organization, we infected *N. benthamiana* leaves with *Tomato golden mosaic virus* (TGMV) prior to the AtWWP1-GFP expression and examined AtWWP1-NB organization in infected cells. As the efficiency of begomovirus infection in epidermal cells is quite low, it is necessary to individually identify infected cells to precisely associate any change in AtWWP1-NB with infection. We used a previously described molecular marker of infected cells, the NSP-mCherry fusion, which is nuclear localized in uninfected cells but moves to the cytosol in infected cells (Carvalho *et al*., 2008c). Therefore, the presence of NSP in the cytosol along with its absence from the nucleus certifies that the cell has been infected in our infection/transient assay. Using the NSP marker of infection, we demonstrated that begomovirus triggers modifications of AtWWP1-NBs during infection, ranging from reorganization to few barely detected NBs to complete dispersal of AtWWP1-NBs in infected cells (Fig. 5). These modifications were observed in the nucleus of 100% of infected cells (total absence of NSP from the nucleus), which contrasted with the typical AtWWP1-NB punctuate organization in uninfected cells (nuclear localized NSP). Even though we did not examine whether viral factors or viral DNA would interact with AtWWP1-NBs in order to exploit these structures for the benefit of the virus, our data support a role of AtWWP1-NBs as components of antiviral defenses against begomoviruses.

Both WW domains are essential for AtWWP1 nuclear body formation and protein dimerization

To map the determinants of nuclear body formation, we generated AtWWP1 deletion fragments and observed their subcellular localization (Fig. 6A and 6B). We also monitored the accumulation of the truncated versions of AtWWP1 by immunoblottings (Fig. 6B). The WW domain regions were required for AtWWP1 to form nuclear bodies. Consistently, fragments comprising only the N-terminus or the C-terminus domains displayed diffused nuclear pattern, but accumulated stably in transient assays (Fig. 6A and 6B). Although the WW domains were the major determinants for NB formation, the truncated protein harboring only the WW domains (AtWWP1$^{193-271}$) formed few nuclear bodies. The truncation AtWWP1$^{193-463}$, which includes both WW domains and the C-terminus portion of the truncated protein, restored full NB-forming activity. Collectively, these results indicated that the AtWWP1-mediated nuclear body organization requires the WW domains and the C-terminus encompasses an accessory domain for enhancing the NB-forming activity.

Many nuclear bodies-forming proteins also harbor the ability to self-associate. Hence, dimerization might be a general feature shared by NB marker proteins (Matera *et al*., 2009). The capacity of AtWWP1 to self-interact was assayed using the yeast-two-hybrid and BiFC interaction assays (Fig. S5). AtWWP1 was fused to AD domain and BD domains of GAL4 and expressed in yeast. The interactions were examined by monitoring His and Ade prototrophy in the presence of 3AT. Similarly, truncated forms of AtWWP1 were generated to identify the region of AtWWP1 responsible for self-association. Our data demonstrated that AtWWP1 forms dimeric structures in yeast and the WW domain regions are necessary for AtWWP1 dimerization (Fig. S5A and S5B). We next used BiFC assays to confirm AtWWP1

dimerization *in planta*. We observed the reconstituted fluorescent signal of YFP in the nucleus of transfected cells. Although a dispersed pattern was also observed, AtWWP1 forms homodimers predominately in NBs (Fig. S5C). Consistent with the yeast two-hybrid assays, the WW domains of AtWWP1 are required for homodimerization *in planta* and for NB-forming activity as well (Fig. S5D). A weak YFP signal was also detected in the nucleus of cells transfected with AtWWP1 and its C-terminus portion ($AtWWP1^{272-463}$), which may indicate another site of interaction. Likewise, the PQBP1 C-terminus segment (residues 220–265) interacts very weakly with the N-terminus segment (residues 1–219; Nabeshima *et al*., 2014). These data indicate that weak intramolecular interactions within full-length protein may be a shared feature between AtWWP1 and PQBP1.

The WW domain harbors two tryptophan residues at conserved positions (Macias *et al*., 2000; Sudol *et al*., 2005). To create a loss-of-function mutant, we prepared a series of mutations by replacing, individually or in combination, the conserved tryptophan residues with alanine (A) in both WW domains. Increasing the number of W-A replacements within the WW domains gradually decreased the NB-forming activity of the mutant proteins (Fig. 7A). The stability of the mutants was monitored by immunoblottings (Fig. 7B). We could not get quadruple mutations by replacing all four conserved W residues with A within the two WW domains. Nevertheless, the triple mutant protein displayed a very low activity and AtWWP1/AWAA nuclear bodies were barely detected in transfected cells. These results demonstrated that the AtWWP1/AWAA mutant is defective for nuclear body formation, although accumulates stably in transient expression assays.

We next examined whether the defective mutant AtWWP1/AWAA retained its capacity to relocate NIG to nuclear bodies in co-localization assays (Fig. 7D). In

100% of co-transfected cells with GFP- and mCherry-fused pairs, NIG was retained in the cytoplasm, whereas the AtWWP1/AWAA mutant localized in the nucleus, as a dispersed pattern with a single nuclear inclusion. Therefore, both co-expressed chimeric proteins kept their typical pattern of localization. Collectively, these results demonstrated that the mutant AtWWP1/AWAA protein failed of relocating NIG to the nuclear bodies. Accordingly, the AtWWP1 triple mutant did not interact with NIG in yeast (Fig. 7C). This loss-of-function mutant is currently being tested in complementation assays upon begomoviruses infection. The eventual goal is to further confirm that the immune activity of AtWWP1 is linked to its NB-forming activity and association with NIG.

AtWWP1 integrates a plant immune system hub network

A well-defined hub form plant immune system network corresponds to CSN5A protein, a catalytic subunit of the COP9 signalosome acting as a key regulator in several basic cellular processes. Interactions between a large set of host immune factors and distinct pathogen effectors from bacteria and fungi have been shown to converge on CSN5A (Mukhtar *et al*., 2011). Consistent with the prediction that effectors from evolutionarily distant pathogens target similar connections in the plant-pathogen interaction network, it has been shown, independently, that the protein AC2 from geminivirus interacts with CSN5A (Lozano-Duran *et al*, 2011). Additionally, NIG and the immune receptor NIK1, both targets of begomovirus NSP, have been shown to also interact with CSN5A (data not shown; Mukhtar *et al*., 2011). To investigate whether AtWWP1 is connected to the immune hub converging to CSN5A, we tested for a potential interaction between AtWWP1 and CSN5A by yeast two-hybrid, Co-IP

and BiFC assays. Co-expression of AD-AtWWP1 fusion and BD-CSN5A fusion in yeast, but not the fusion proteins with the reciprocal empty vector, activated the *HIS3* reporter gene and promoted prototrophy in the presence of 3AT (Fig. 8A). These interactions were examined *in planta* by CoIP (Fig. 8B). Total protein was extracted from *N. benthamiana* leaves transiently expressing AtWWP1-6HA and/or CSN5A-GFP and incubated with Anti-GFP antibodies-coupled magnetic beads. Anti-GFP co-immunoprecipitated AtWWP1-6HA, which was detected using an HA-antibody, confirming a previous association between the recombinant proteins (Fig. 8B).

We next used BiFC assays to further confirm the interaction between AtWWP1 and CSN5A *in planta*. YFP fluorescence was reconstituted in cells co-infiltrated with constructs corresponding to nYFP-CSN5A and cYFP-AtWWP1 (Fig. 7C). We observed that the interaction between CSN5A and AtWWP1 occurs in the nucleus, displaying a diffused pattern. Co-expression of CSN5A or AtWWP1 with the respective empty vectors did not restore the YFP fluorescence. Since CSN5A and AtWWP1 interact in the nucleus, we examined whether CSN5A co-localizes with AtWWP1 in nuclear bodies. No detectable co-localization signal was observed in nuclear bodies, despite to the visualization of an overlapping signal at the nucleoplasmic region (Fig. 5D). We concluded that AtWWP1 interacts with CSN5A in the nucleus, but do not co-localize with AtWWP1 in nuclear bodies. Collectively, these data suggest that AtWWP1 may be a component of the plant immune system linked to the CSN5A immune hub.

Discussion

Cytosolic NIG may be relocated to the nucleus under certain stimuli

The geminivirus nuclear shuttle protein (NSP) facilitates the intracellular transport of viral DNA from the nucleus to the cytoplasm and acts along with the movement protein (MP) to translocate the viral DNA to adjacent cells. The mechanism by which NSP mediates the nucleocytoplasmic movement of the viral DNA is still unknown. We have previously shown that the NSP-interacting GTPase (NIG), which is localized around the nuclear envelope at the cytosolic side, facilitates the translocation of NSP-DNA complexes from the nucleus to the cytoplasm and may act as a cellular cofactor for NSP function**.** In spite of presenting biochemical and structural properties consistent with a role in the nucleocytoplasmic transport of protein complexes, the NIG cellular function remains to be elucidated. In this work, we identified a cellular partner of NIG, a nuclear WW domain-containing protein designated AtWWP1, which forms nuclear bodies (Fig 1 and Fig 2). When both proteins are co-expressed, NIG is relocated to the nucleus where it interacts with AtWWP1 in nuclear bodies (Fig. 3). These data show that NIG can dynamically changes its subcellular localization, endorsing the previous hypotheses that NIG may participate in nucleocytoplasmic trafficking of molecules (Carvalho *et al*., 2008b).

AtWWP1 is part of a plant antiviral immunity mechanism via nuclear inclusions

Although the interaction AtWWP1-NIG did not uncovered the cellular function of NIG, we provided several lines of evidence indicating that AtWWP1 displays an

antiviral function. First, virus infection increased the expression level of *AtWWP1*. Second, loss of *AtWWP1* function enhanced susceptibility to begomovirus infection. The *atwwp1* knockout line displayed more severe symptoms of begomovirus, an accelerated course of infection and a tendency to accumulate more viral DNA as compared to wild-type lines. Third, a tolerant phenotype displayed by *AtWWP1*-overexpressing lines correlated with the expression level of the transgene. Fourth, AtWWP1 might disrupt the NIG pro-viral function by sequestering NIG in nuclear bodies apart from its typical cytosolic localization. Fifth, begomovirus triggers modifications of AtWWP1-NBs during infection, resembling the behavior of mammalian immune NBs during virus infection (Gu and Zheng, 2016). Finally, AtWWP1 was dynamically integrated into the conserved CSN5A immune hub as it interacted directly with CSN5A in a nuclear body-disrupting manner.

Recently, antiviral functions have been associated with WW domain-containing proteins, which displays an inhibitory role during RNA virus infection (Barajas *et al*., 2015). The Nedd4-type Rsp5p E3 ubiquitin ligase, Wwm1p and Prp40 from yeast inhibit *Tomato bushy stunt virus* (TBSV) replication, which is the type species of the *Tombusviridae* family. Additionally, Arabidopsis AtDrh1, AtFCA, and AtPRP40C, all of them harboring WW domain, inhibited *Cucumber necrosis virus* (CNV, a very close relative of TBSV) genomic RNA accumulation (Barajas *et al*., 2009; Qin *et al*., 2012). The proposed hypothesis is that host WW domains bind to the viral replication protein p33 and inhibits its ability to bind to the viral RNA and to the other p92 replication protein, impairing tombusviral replication (Barajas *et al*., 2015). Moreover, WW domain-containing proteins may facilitate the degradation of excess amounts of viral replication proteins (Nagy, 2015). Yeast Rsp5 has an inhibitory role in tombusvirus replication by decreasing p92 replication protein levels

via protein degradation (Barajas *et al*., 2009). Differently from Arabidopsis AtDrh1, AtFCA, and AtPRP40C, AtWWP1 did not show an inhibitory effect in TBSV replication, neither interacted with p33 viral protein (Barajas *et al*., 2009), which suggests that AtWWP1 does not participate in RNA antiviral immunity. A shared feature of DNA virus is the need to enter in the nucleus for replication and gene expression and to shuttle back to the cytoplasm to invade the adjacent cells via plasmosdesma. NIG may act specifically at this step of *Begomovirus* infection pathway, favoring the movement of viral DNA from nucleus to cytoplasm. The AtWWP1-mediated relocation of NIG from the cytoplasm to nuclear bodies may disrupt the pro-viral activity of NIG; thereby compromising begomovirus infection. Consistently with our hypothesis, AtWWP1 antiviral immunity may be specific to DNA virus, once it targets the NSP-interacting hub. Therefore, AtWWP1 may provide a spatial regulation of NIG inside the nucleus, sequestering NIG into nuclear bodies, which may perturb the NIG function by preventing its association with viral NSP (Fig. 9).

Arabidopsis proteins exhibiting nuclear bodies-forming activity have been reported to be involved in plant immunity. AtMORC1 (CRT1), identified as a hyper-sensitive mutant to *Turnip crinkle virus*, forms nuclear bodies and is involved in plants defense (Kang *et al*., 2008; Harris *et al*., 2016). Similarly, AtMORC4 and AtMORC7 also exhibit the capacity to form nuclear bodies, to act as repressor of genes involved in pathogen response and functions redundantly in defense against Emwa1 isolate of the oomycete pathogen, *Hyaloperonospora arabidopsidis* (Hpa; Harris *et al*., 2016). Our data suggest that the antiviral role displayed by AtWWP1 may be linked to its nuclear bodies-forming capacity. In fact, AtWWP1 may suppress the pro-viral function of NIG by sequestering this host factor into nuclear bodies, which impairs its

cytosolic transport function. Furthermore, the AtWWP1-NB organization is dynamically modified by begomovirus infection. Finally, AtWWP1 interacts with CSN5A, a central component of a conserved immune hub, in a NB-disrupting manner, which may compromise an intrinsic antiviral defense. CSN5A has been described as a central conserved hub in the plant immune system network to which converge the interactions of pathogen effectors from divergent pathogens (Mukhtar *et al*., 2011). Accordingly, the AC2 from geminivirus and the immune receptor NIK1, which is a target of the begomovirus NSP, have been shown to interact with CSN5A (Lozano-Duran *et al*, 2011; Fig. S6), implicating the CSN5A hub as a functional component of the geminivirus-host interactome. The disorganization of the AtWWP1 nuclear bodies upon CSN5A interaction and begomovirus infection may resemble the dynamic structure of the mammalian promyelocytic leukemia (PML) nuclear bodies, which play a role in antiviral innate immunity and are frequently modified during viral infection (Gu and Zheng, 2016). These modifications antagonize a direct antiviral function of individual PML-NB components, which act as restrictive factors against DNA viruses. We have demonstrated that the AtWWP1/AWAA mutant, which compromises its full NB-forming capacity, also blocks the capacity to relocate NIG to nuclear bodies (Fig. 7). Although the AtWWP1 nuclear body-forming activity and heterodimerization activity seems to be uncoupled, the examination of the antiviral activity of the NB-forming deficient mutant will help defining whether the antiviral activity of AtWWP1 is also linked to the AtWWP1 nuclear bodies or simply to its capacity of redistributing NIG to the nucleus.

The AtWWP1 nuclear bodies may display a regulatory role in transcription

AtWWP1 is an uncharacterized protein, which contains two WW domains (http://pfam.xfam.org/; Fig S1). It is conserved in plant species, including agronomic crops *Glycine Max, Oriza sativa* and *Solanum lycopersicum* (Fig. S1). In Arabidopsis, AtWWP1 is closely related to AtPrp40s (Fig S1), a group of splicing factors that interacts with the CTD domain of RNA polymerase II (CTD-RNAPII). Besides the WW domains, AtPrp40s possess FF domains, characterized by two highly conserved phenylalanine residues (Kang *et al*., 2009). Differently from yeast Prp40 in which both WW and FF domains function as CTD-RNAPII -binding domains, in Arabidopsis, Prp40s interaction with CTD-RNAPII is mediated by the WW domain only (Kang *et al*., 2009). Sequence similarities with AtPrp40s raised up a possible connection between AtWWP1 and transcriptional process, trough association with RNA polymerase II via its WW domain. Consistent with this hypothesis, the AtWWP1 human homologous PQBP1 displays distinct role in pre-mRNA splicing and transcription. PQBP1 has previously been show to interact with RNA polymerase II and the transcription factor Brn-2 (Waragai *et al*., 1999; Okazawa *et al*., 2002). Additionally, PQBP1 is associated with splicing factors, including as SIPP1 (also known as WBP11 and NpwBP) and U5-15KD, through its WW domain and C-terminus domain, respectively (Komuro *et al.,* 1999; Waragai *et al*., 2000; Mizuguchi *et al*., 2014). Interestingly, SIPP1 is a nucleocytoplasmic shuttling splicing factor that co-localizes with PQBP1 in nuclear inclusion bodies (Liorian *et al*., 2005). Likewise, co-expression of PQBP1 and its U5–15 kDa ligands or ataxin-1 also resulted in nuclear bodies co-localization (Okasawa *et al*. ,2002;). Sequence analysis revealed that AtWWP1 and PQBP1 shares a C-terminal conserved region, in addition to WW

domain (Fig. S1). Collectively, these data strengthen the hypothesis AtWWP1 forms transcriptionally active nuclear bodies.

Subnuclear compartimentalization of transcriptional machinery have been reported in higher eukaryotes (Papantonis and Cook, 2013; Allen and Taatjes, 2015). AtWWP1 forms dynamic subnuclear bodies of diverse size and number through its WW domains (Fig. 2 and Fig. 6). Because the WW domain mediates the interaction with several factors, AtWWP1 could modulate transcription trough recruitment of proteins into nuclear bodies.

Nuclear bodies are dynamic nuclear domains, which provides a specialized environment for a variety of biological processes like DNA replication and repair, gene expression, splicing and protein degradation (Shaw and Brown, 2004; Mao *et al*., 2011). In plants, these subnuclear structures have been implicated in several hormone- and light-regulated signaling pathways, as well as with cicardian clock components. Nuclear bodies can sequester and release specific factors; thereby, regulating their concentration in the nucleus, according to the cell physiological *status* (Dundr, 2012). NIG interacts and co-localizes with AtWWP1 in nuclear bodies (Fig.1 and Fig. 3). Although the functional relevance of the NIG nuclear translocation is unclear, it is possible to be a mechanism to control NIG stability in the cell. Therefore, AtWWP1 might control NIG stability and regulate its function trough subnuclear sequestration. In fact, spatial regulation, such as nuclear translocation and sub-compartmentalization, has been previously reported. In plants, GIGANTEA, a key component of cicardian clock, seems to be spatially regulated through its physical association with EFL4 (EARLY FLOWERING 4) in nuclear bodies. Sequestering GIGANTEA in nuclear bodies restricts its access to CONSTANS promoter and contributes to the regulation of photoperiodic flowering (Kim *et al*., 2013).

Photobodies are one of the most well characterized plant nuclear bodies (Chen, 2008; Van Buskirk *et al*., 2012). These micro domains represent an active site of gene expression regulation, at the level of transcription, and may also act at post-transcriptional level, like the involvement of phytochrome controlling alternative splicing in Arabidopsis (Shikata *et al*., 2014). Photobodies display dynamic patterns in plant cells, varying from diffused to punctuate patterns whose size, number and potentially function may change in response to different light conditions (Chen *et al*., 2003; Chen and Chory, 2011). Collectively, our data suggest that AtWWP1 nuclear bodies may act as an active site for transcriptional regulation. Further experiments will allow us to elucidate the *in vivo* function of AtWWP1 as well its nuclear body dynamics.

Material and methods

Plasmid constructions

All recombinant plasmids were generated by GATEWAY cloning system (Invitrogen, http://www.invitrogen.com/). Primers used for the amplification of genes, described in this study, are listed at the Supplemental Table 2 (Table S2). *AtWWP1* (AT2G41020) and *CSN5A* (AT1G22920) coding regions either with (ST) or without (NS) a translational stop codon were amplified from cDNA of Arabidopsis ecotype Columbia and inserted into the entry vectors pDONR201 and pDONR207. The resulting clones were designated AtWWP1ST-pDONR201 (pUFV1427), AtWWP1NS-pDONR201 (pUFV1434), CSN5AST-pDONR201 (pUFV1435), CSN5ANSp-DONR201 (pUFV1436), AtWWP1NS-pDONR207 (pUFV1644) and CSN5ANS-

pDONR207 (pUFV1645). Full-length NIG and its Proline-Rich domain-derived DNA constructions were obtained previously (Carvalho *et al*., 2008). The amplified products were cloned into entry vectors and transferred to different destination vectors for expression in yeast and plants. The NIG- and Proline Rich-derived clones used in this investigation have been previously described and designated as NIGST-pDONR207 (pUFV1083), NIGNS-pDONR207 (pUFV1643) and PRNIG-pDEST32 (pUFV1098).

For yeast two-hybrid assay, AtWWP1 and CSN5A coding regions were transferred from the respective entry clones (AtWWP1ST-pDONR201 and CSN5AST-pDONR201) to the yeast expression vectors pDEST32 and pDEST22, generating the clones pBD-AtWWP1 (pUFV1474), pAD-AtWWP1 (pUFV1483), pAD-CSN5A (pUFV1482) and pBD-CSN5A (pUFV1488), as GAL4 binding domain (BD) or activation domain (AD) fusions.

For subcellular localization and co-immunoprecipitation assays, AtWWP1, CSN5A and NIG coding regions were fused to fluorescent tags GFP and mCherry as well as to haemagglutinin (HA) tag and expressed in plants driven by 35S CaMV promoter. The resulting recombinant constructions were 35S::AtWWP1-GFP (pUFV1448), 35S::CSN5A-GFP (pUFV1447), 2x35S::AtWWP1-mCherry (pUFV2179), 2x35S::CSN5A- mCherry (pUFV2222), 2x35S::NIG- mCherry (pUFV2220), 2x35S::AtWWP1-6HA (pUFV1948), 2x35S::CSN5A- 6HA (pUFV1947), 2x35S::NIG-6HA (pUFV1946). 35S::YFP-NIG has been already described (Carvalho *et al*., 2008).

The vectors SPYNE-GW and SPYCE-GW, which contain the N-terminal (nYFP) or C terminus (cYFP) region of YFP, respectively, were used in BIFC experiments. AtWWP1NS-pDONR207, CSN5ANS-pDONR207 and NIGNSp-

DONR207 (pUFV1643) were transferred to SPYNE-GW and SPYCE-GW resulting in AtWWP1-nYFP (pUFV1650), AtWWP1-Cyfp (pUFV1651), CSN5A-nYFP (pUFV1648), CSN5A-cYFP (pUFV1649), NIG-nYFP (pUFV1646), NIG-cYFP (pUFV1647) clones.

Truncated versions of AtWWP1 were obtained from AtWWP1ST-pDONR201 by PCR isolation using specific primers listed in Table S2. The generated fragments were cloned into the entry vector pDONR207 and named as AtWWP1$^{1\text{-}193}$NS-pDONR207, AtWWP1$^{1\text{-}271}$NS-pDONR207, AtWWP1$^{193\text{-}271}$NS-pDONR207, AtWWP1$^{193\text{-}361}$NS-pDONR207, AtWWP1$^{193\text{-}463}$NS-pDONR207, AtWWP1$^{272\text{-}463}$NS-pDONR207 and AtWWP1$^{362\text{-}463}$NS-pDONR207. The inserts from these entry vectors were then transferred to different vectors for expression in plants (pK7FWG2, SPYNE-GW and SPYCE-GW). The obtained clones AtWWP1$^{1\text{-}193}$-GFP, AtWWP1$^{1\text{-}271}$-GFP, AtWWP1$^{\text{-}193\text{-}271}$-GFP , AtWWP1$^{193\text{-}361}$-GFP, AtWWP1$^{193\text{-}463}$-GFP, AtWWP1$^{272\text{-}463}$-GFP and AtWWP1$^{362\text{-}463}$-GFP as well as AtWWP1$^{1\text{-}193}$-nYFP, AtWWP1$^{1\text{-}271}$-nYFP, AtWWP1$^{193\text{-}271}$-nYFP, AtWWP1$^{193\text{-}361}$-nYFP, AtWWP1$^{193\text{-}463}$-nYFP, AtWWP1$^{272\text{-}463}$-nYFP and AtWWP1$^{362\text{-}463}$-nYFP, as AtWWP1$^{1\text{-}193}$-cYFP, AtWWP1$^{1\text{-}271}$-cYFP, AtWWP1$^{193\text{-}271}$-cYFP, AtWWP1$^{193\text{-}361}$-cYFP, AtWWP1$^{193\text{-}463}$-cYFP, AtWWP1$^{272\text{-}463}$-cYFP and AtWWP1$^{362\text{-}463}$-cYFP, were used for transient expression in *N. benthamiana* leaves. In order to perform yeast two-hybrid assay, truncated versions of AtWWP1 were also cloned in the yeast expression vector pDEST22, resulting in pAD-AtWWP1$^{1\text{-}193}$, pAD-AtWWP1$^{1\text{-}271}$, pAD-AtWWP1$^{193\text{-}271}$, pAD-AtWWP1$^{193\text{-}361}$, pAD-AtWWP1$^{193\text{-}463}$, pAD-AtWWP1$^{272\text{-}463}$ and pAD-AtWWP1$^{362\text{-}463}$.

To generate AtWWP1 fusions driven by its native promoter, 2-kb 5' flanking sequence of the *AtWWP1* gene was PCR-amplified using the primers described in

Table S1 and cloned into pDONR-P4-P1R. The 2-kb *AtWWP1* 5' flanking sequence was then transferred by triple recombination to the destination vector pK7m34GW along with AtWWP1-pDON201 and YFP-pDONR-P2R-p3, yielding the clone pAtWWP1::AtWWP1-YFP.

For GUS histochemical assay, around 1000bp of the 5' flanking sequences of *AtWWP1 and NIG* were amplified from Arabidopsis genomic DNA using Taq Platinum and specific oligonucleotides (Table S2) and inserted into the entry vector pCR8/GW/TOPO (Life Techonology; pUFV1802). The promoter sequences were then transferred by recombination into the destination vector pMDC162. The resulting clones pAtWWP1-MDC162 (pUFV1803) and pNIG-MDC162 (pUFV2022), harbor the respective promoter sequences of AtWWP1 and NIG fused to the β-glucuronidase (GUS) reporter gene.

Phylogenetic analysis

The amino acid sequences were recovered from TAIR (http://arabidopsis.org/) and Phytozome v11 databases. The WW domain was predicted with HMMER software using the pfam v.30 database. The amino acid sequences containing WW domain were aligned using MUSCLE. Phylogenetic trees were constructed using Bayesian inference performed with MrBayes v3.2.2 with mixed amino acid substitution model (Jones). The analyses were carried out running 20.000.000 generations and excluding the first 5.000.000 generations as burn-in. Plant AtWWP1 homolog proteins were aligned with ClustalW (http://www.ebi.ac.uk/clustalw/index.html)

AtWWP1 mutagenesis

Mutagenesis of AtWWP1 were performed based on the QuickChange™ site-directed mutagenesis protocol (Stratagene) using the recombinant plasmid AtWWP1-NSpDONR207 as template. The PCR amplifications were carried out using 2U of Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific), 50 ng of template, 2 mM primer pair (Table S2) and 200 mM dNTPs in 50 µL total reaction. The PCR-amplification mutagenized products were cloned in the entry vector resulting in the single mutation clones AtWWP1^{W198A}NS-pDONR207 (pUFV2997) and AtWWP1^{W243A}NS-pDONR207 (pUFV2998). The resulting clones containing single mutations were used to generate double and triple mutants: AtWWP1^{W198AW221A}NS-pDONR207 (pUFV2795), AtWWP1^{W243AW266A}NS-pDONR207 (pUFV2999) and AtWWP1^{W198AW243AW266A}-NSpDONR207 (pUFV3000). The resulting inserts were cloned in the plant expression vectors pk7FWG2 or pEarleyGate103 and named: AtWWP1^{W198A}-GFP, AtWWP1^{W243A}-GFP, AtWWP1^{W198AW221A}-GFP, AtWWP1^{W243AW266A}-GFP and AtWWP1^{W198AW243AW266A}-GFP (pUFV2796).

For yeast two-hybrid experiments, the AtWWP1 mutant sequences were inserted in pDEST22 resulting in pAD-AtWWP1^{W198A} (pUFV2772), pAD-AtWWP1^{W243A} (pUFV2771), pAD-AtWWP1^{W198AW221A} (pUFV2795), pAD-AtWWP1^{W243AW266A} (pUFV2773) and pAD-AtWWP1^{W198AW243AW266A} (pUFV2774) clones.

Plant material, growth conditions and transformation

The Columbia (Col-0) ecotype of *A. thaliana* was used as the wild-type. Homozygous seeds of the T-DNA insertion *atwwp1* mutant (Salk_073780) were obtained from the Arabidopsis Biological Resource Center. Arabidopsis seeds were

surfaced sterilized and sown on Murashige and Skoog (MS)-containing agar plates supplemented with sucrose. Plates were stratified in the dark at 4°C for 72 hours. Plants were grown in a growth chamber at 22 ºC under long-day conditions (16 h light/8 h dark). Stable transgenic lines were obtained via *A. tumefaciens*–mediated transformation using a floral dip method (Zhang *et al*., 2006). Plants transformed with the indicated constructions were selected on MS medium supplemented with antibiotics. The selected transformants were transferred into soil, and the transgenic lines were confirmed using PCR.

Yeast two-hybrid assay

Saccharomyces cerevisiae AH109 strain was co-transformed with the yeast expression vectors pDEST22 (Gal4 AD) and pDEST32 (Gal4 DB) harboring the coding region of tested proteins by the lithium acetate/polyethylene glycol method. Transformed yeast cells bearing both plasmids were selected on synthetic dropout medium (SD) lacking Trp, Leu for 4 days at 28 °C. Specific interactions were tested by growing transformed cells on SD medium lacking Trp, Leu, His or Trp, Leu, His, supplemented with 3-amino-1,2,4-triazole (1 to 10 mM) to test the strength of the interaction. Quantitative measurements of β-galactosidase activity in yeast extracts were monitored using o-nitrophenyl β-D-galactopyranoside (ONPG) assay, as described previously (Uhrig *et al*., 1999).

Co-immunoprecipitation assay

The *in vivo* interactions between AtWWP1 and its cellular partners NIG and CSN5A were assayed by co-immunoprecipitation using the μMACS Epitope Tag

Protein Isolation Kit (MACS/Miltenyi Biotec), according to the manufacturer's instructions. Transient *Agrobacterium*-mediated expression in *N. benthamiana* leaves was performed, as previously described by Carvalho *et al*. 2008. Total protein extracts were obtained from infiltrated leaves expressing the tested recombinant proteins and incubated for 2 h with anti-GFP magnetic beads (MACS/Miltenyi Biotec) at 4 ºC under gentle rotation. After the incubation step, the extracts were applied into a MACS column placed in the indicated separator and washed five times for removal of unbound material. The elution step was performed using 50 µL of elution buffer pre-warmed to 95 ºC. Eluted fractions were resolved on a 10% (w/v) SDS-PAGE gel, immunoblotted with anti-HA (Miltenyi Biotec, catalogue number 130-091-972) or anti-GFP (Miltenyi Biotec, catalogue number 130-091-833) monoclonal antibodies. The reacting antibodies were detected using Signal West Pico Chemiluminescent Substrate (Thermo Scientific) according to the manufacturer's instructions.

Bimolecular fluorescence complementation (BiFC)

Different combinations of the *A. tumefaciens* strain GV3101 expressing the indicated NYFP/CYFP-fused proteins were co-infiltrated into the abaxial surface of *N. benthamiana* leaves at an OD_{600nm} ratio of 1:1: for infiltration. Fluorescence was analyzed in epidermal cells 3 days after infiltration using a Zeiss inverted LSM510 META laser scanning microscope equipped with an argon laser and a helium laser as excitation source. YFP was excited at 514nm using an argon laser, and YFP emission was detected using a 560-615nm filter.

Subcellular Localization

Subcellular and subnuclear localizations of fluorescence fusion proteins were analyzed by transient expression in *N. benthamiana* leaves. *A. tumefaciens* GV3101 was transformed with the tested protein fused to GFP, YFP or mCherry fluorescent tags. Fluorescence was visualized in epidermal cell layers of the leaves after 3 day of infiltration using a confocal microscopy. Stable transgenic lines expressing the recombinant fluorescent proteins were also assayed for subcellular localization. Root fragments from different Arabidopsis transgenic seedlings were imaged by confocal microscopy. For imaging GFP, the 488nm excitation line and the 500 to 530nm band pass filter were used. Excitation of YFP was at 514nm and YFP emission was detected by using a 560–615nm filter. Excitation of mCherry was at 540nm and emission of 608-680nm.

CaLCuV infection assay

Arabidopsis *AtWWP1*-overexpressing transgenic lines, a T-DNA-insertion mutant (*atwwp1*) and Columbia wild type were challenged by *Cabbage leaf curl virus* (CaLCuV) infection. Six-leaf stage Arabidopsis plants were inoculated with tandemly repeated CaLCuV DNA-A and DNA-B by biolistic delivery using a microprojectile bombardment model PDS-1000/He accelerator at 900 psi. The inoculated plants were transferred to a growth chamber and examined for symptom development and the course of infection was monitored, as previously described (Florentino *et al*., 2006). Total nucleic acid was extracted from systemically infected leaves and viral DNA was detected by PCR with DNA-A or DNA-B begomovirus-specific primers PBL1v2040 and PCRC1 (Rojas *et al*.,1993; Table S2)

Quantification of viral DNA in infected plants

Viral DNA accumulation in infected plants was quantified by qPCR, as previously described (Zorzatto *et al*., 2015). 100 ng of total nucleic acid from systemically infected leaves were included in 10 µL mix reaction containing the Fast SYBR GreenMasterMix (Life Technologies) and specific CaLCuV-B primers (Table S1). Samples were amplified on a 7500 Real Time PCR System (Applied Biosystems). The genomic copies of CaLCuV were normalized against an internal control (18S rRNA) to consider template input variation between tubes. Standard curves of viral and host DNA, which were calculated based in regression analysis of the respective Ct values, were used as reference control.

Quantitative Real-Time PCR analysis

Primer pairs for real-time PCR were designed using Primer Express 3.0 (Life Technologies). Total RNA was extracted from Arabidopsis leaves using TRIzol (Thermo Fisher Scientific). Two microgram of total RNA were used for cDNA synthesis using oligo (dT) primers (0.5 µM) and 1U of M-MLV reverse transcriptase (Invitrogen Life Technologies), as previously described (Delú-Filho *et al*., 2000). For real-time PCR, the reaction mixture consisted of cDNA template, primer mix (5 mmol each) and SYBR Green PCR Master Mix (Life Technologies) in a total volume of 10µl. Real-time RT-PCR assays were performed on an ABI7500 instrument (Life Technologies).

The amplification reactions were performed as follows: 2 min at 50 ºC, 10 min at 95 ºC and 40 cycles of 94 ºC for 15 s and 60 ºC for 1 min. Actin was used as an internal control to normalize all values in the real-time RT-PCR assays. Gene expression was quantified using the $2^{-\Delta Ct}$ method.

Promoter GUS assay

The histochemical analysis of β-glucuronidase activity was performed according to McCabe *et al*. 1988. Three independent transgenic lines transformed with pAtWWP1-MDC162 or pNIG-MDC162 construction were germinated and grown on MS medium as described above. Seedlings were embedded in the GUS assay buffer [100 mM NaH_2PO_4.H2O (pH 7.0), 0.5 mM $K_4Fe(CN)_6.3H_2O$, 10 mM $Na_2EDTA.2H_2O$, 0.1% (v/v) Triton X-100] containing 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (X-Gluc; McCabe *et al*., 1988) and incubated at 37 ºC in the dark for 16 h. Pigments were extracted from stained tissues with methanol:acetone (3:1). The image of the stained seedlings was obtained by a digital camera attached to a stereomicroscope.

AtWWP1 nuclear bodies stability under Begomovirus infection

Tobacco leaves were inoculated with partial tandem repeats of *Tomato golden mosaic virus* (TGMV) DNA-A and DNA-B by biolistic delivery, as described previously (Fontes *et al*., 2004; Carvalho *et al*., 2008c). 14 days after inoculation, after TGMV infection confirmation, tobacco leaves were co-infiltrated with AtWWP1-GFP and NSP-mCherry constructions. Leaf fragments were analyzed 3 days after infiltration using a confocal microscope.

References

Allen, B.L.,Taatjes, D.J. (2015) The Mediator complex: a central integrator of transcription. *Nat. Rev. Mol. Cell Biol*. 16,155-166.

Barajas, D., Kovalev, N., Qin, J., Nagy, P.D. (2015) Novel mechanism of regulation of tomato bushy stunt virus replication by cellular WW domain proteins. *J. Virol*. 89, 2064–2079.

Barajas, D., Li, Z., Nagy, P.D. (2009) The Nedd4-type Rsp5p ubiquitin ligase inhibits tombusvirus replication by regulating degradation of the p92 replication protein and decreasing the activity of the tombusvirus replicase. *J. Virol*. 83, 11751–11764.

Becerra, S., Andrés-León, E., Prieto-Sánchez, S., Hernández-Munain, C., Suñé, C. (2016) Prp40 and early events in splice site definition. *Wiley Interdiscip. Rev. RNA*. 7, 17-32.

Calil, I.P. , Fontes, E.P.B. (2016) Plant immunity against viruses: antiviral immune receptors in focus. *Ann. Bot.* 118, 200-213.

Carvalho, C.M., Fontenelle, M.R., Florentino, L.H., Santos, A.A., Zerbini, F.M., Fontes, E.P.B. (2008a) A novel nucleocytoplasmic traffic GTPase identified as a functional target of the bipartite geminivirus nuclear shuttle protein. *Plant J.* 5, 869-880.

Carvalho, C.M., Machado, J.P.B., Zerbini, F.M., Fontes, E.P.B. (2008b) NSP-interacting GTPase: A cytosolic protein as cofactor for nuclear shuttle proteins. *Plant Signal. Behav.* 3, 752-754.

Carvalho, C.M., Santos, A.A., Pires, S.R., Rocha, C.S., Saraiva, D.I., Machado, J.P., Mattos, E.C., Fietto, L.G. and Fontes, E.P.B. (2008c) Regulated nuclear trafficking of rpL10A mediated by NIK1 represents a defense strategy of plant cells against virus. *PLoS Pathog*. 4, e1000247.

Chen, M. (2008) Phytochrome nuclear body: an emerging model to study interphase nuclear dynamics and signaling. *Curr. Opin. Plant Biol*. 11, 503–508.

Chen, M., Chory, J. (2011) Phytochrome signaling mechanisms and the control of plant development. *Trends Cell Biol*. 21, 664–671.

Chen, M., Schwab, R., Chory, J. (2003) Characterization of the requirements for localization of phytochrome B to nuclear bodies. *Proc. Natl. Acad. Sci. USA*. 100, 14493–14498.

Dundr, M. (2012) Nuclear bodies: multifunctional companions of the genome. *Curr. Opin. Cell Biol*. 24, 415–422.

Florentino, L.H., Santos, A.A., Fontenelle, M.R., Pinheiro, G.L., Zerbini, F.M., Baracat-Pereira, M.C., Fontes, E.P.B. (2006) A PERK-Like receptor kinase interacts with the geminivirus nuclear shuttle protein and potentiates viral infection. *J. Virol*. 80, 6648-6656.

Fondong, V.N. (2013) Geminivirus protein structure and function. *Mol. Plant Pathol*. 14, 635-649.

Fontes, E.P.B., Gladfelter, H.J., Schaffer, R.L., Petty, I.T.D., Hanley-Bowdoin, L. (1994) Geminivirus replication origins have a modular organization. *Plant Cell.* 6, 405–416.

Fontes, E.P.B., Santos, A.A., Luz, D.F., Waclawovsky, A.J., Chory, J. (2004) The geminivirus nuclear shuttle protein is a virulence factor that suppresses transmembrane receptor kinase activity. *Genes Dev*. 18, 2545-2556

Gafni, Y., Epel, B.L. (2002) The role of host and viral proteins in intra and inter-cellular trafficking of geminiviruses. *Physiol. Mol. Plant Pathol*. 60, 231–241.

Gouveia, B.C.C, Calil, I.P., Machado, J.P.B., Santos, A.A., Fontes, E.P.B. (2016) Immune Receptors and Co-receptors in Antiviral innate immunity in plants. *Front. Microbiol.* 7, 2139.

Gu, H., Zheng, Y. (2016) Role of ND10 nuclear bodies in the chromatin repression of HSV-1. *Virol. J.* 13, 62-70.

Hanley-Bowdoin, L., Bejarano, E.R., Robertson, D., Mansoor, S. (2013) Geminiviruses: masters at redirecting and reprogramming plant processes. *Nat. Rev. Microbiol*. 11, 777–88.

Harris, C.J., Husmann, D., Liu, W., Kasmi, F.E., Wang, H., Papikian, A., Pastor, W.A., Moissiard, G., Vashisht, A.A., Dang, J.L., Wohlschlegel, J.A., Jacobsen, S. E. (2016). Arabidopsis AtMORC4 and AtMORC7 form nuclear bodies and repress a large number of protein-coding genes. *PLoS Genet. 12*(5), e1005998.

Ingham, R.J., Colwill, K., Howard, C., Dettwiler, S., Lim, C.S, Yu, J., Hersi, K., Raaijmakers, J., Gish, G., Mbamalu, G., Taylor, L., Yeung, B., Vassilovski, G., Amin, M., Chen, F., Matskova, L., Winberg, G., Ernberg, I., Linding, R., O'donnell, P., Starostine, A., Keller, W., Metalnikov, P., Stark, C., Pawson, T. (2005) WW

domains provide a platform for the assembly of multiprotein networks. *Mol. Cell Biol*. 25, 7092–106.

Inoue-Nagata, A.K., Lima, M.F., Gilbertson, R.L. (2016) A review of geminivirus (begomovirus) diseases in vegetables and other crops in Brazil: current status and approaches for management. *Hort. Brasil.* 34, 8-18.

Kang, C.H., Feng, Y., Vikram, M., Jeong I.S., Lee, J.R., Bahk, J.D., Yun, D.J., Lee, S.Y., Koiwa H. (2009) *Arabidopsis thaliana* PRP40s are RNA polymerase II C-terminal domain-associating proteins. *Arch. Bioch. Biophysics*. 484, 30-38.

Kang, H.G., Kuhl, J.C., Kachroo, P., Klessig, D.F. (2008) CRT1, an Arabidopsis ATPase that interacts with diverse resistance proteins and modulates disease resistance to turnip crinkle virus. *Cell Host Microbe*, 3, 48–57.

Kao, H.Y., Siliciano, P.G. (1996) Identification of Prp40, a novel essential yeast splicing factor associated with the U1 small nuclear ribonucleoprotein particle. *Mol. Cell Biol*. 16, 960–967.

Kim, Y., Lim, J., Yeom, M., Kim, H., Kim, J., Wang, L., Kim, W.Y., Somers, D.E., Nam, H.G. (2013) ELF4 regulates GIGANTEA chromatin access through subnuclear sequestration. *Cell Rep*. 3, 671–677.

Komuro, A., Saeki, M., Kato, S. (1999) Association of two nuclear proteins, Npw38 and NpwBP, via the interaction between the WW domain and a novel prolinerich motif containing glycine and arginine. *J. Biol. Chem*. 274, 36513–36519.

Kunde, S.A., Musante, L., Grimme, A., Fischer, U., Muller, E., Wanker, E.E., Kalscheuer, V.M. (2011) The X-chromosome-linked intellectual disability protein PQBP1 is a component of neuronal RNA granules and regulates the appearance of stress granules. *Hum. Mol. Genet*. 20, 4916–4931.

Lazarowitz, S.G., Beachy, R.N. (1999) Viral movement proteins as probes for intracellular and intercellular trafficking in plants. *Plant Cell*, 11, 535–548.

Llorian, M., Beullens, M., Lesage, B., Nicolaescu, E., Beke, L., Landuyt, W., Ortiz, J.M., Bollen, M. (2005) Nucleocytoplasmic shuttling of the splicing factor SIPP1. *J. Biol. Chem*. 280, 38862–38869.

Lozano-Durán, R., Rosas-Díaz, R., Gusmarolli, G., Luna, A.P., Taconat, L., Deng, X.W., Benajaro, E.R. (2011) Geminiviruses subvert ubiquitination by altering CSN-mediated derubylation of SCF E3 ligase complexes and inhibit jasmonate signaling in *Arabidopsis thaliana*. *Plant Cell*. 23, 1014-1032.

Machado, J.P.B., Calil, I.P., Santos, A.A., Fontes, E.P.B. (2017) Translational control in plant antiviral immunity. *Gen. Mol. Biol.* 40, 1-13.

Macias, M.J., Wiesner, S., Sudol, M. (2000) WW and SH3 domains, two different scaffolds to recognize prolinerich ligands. *FEBS Lett*. 513, 30–37.

Mao, Y.S., Zhang, B., Spector, D.L. (2011) Biogenesis and function of nuclear bodies. *Trends Genet.* 27, 295–306.

Matera, A.G., Izaguire-Sierra, M., Praveen, K., Rajendra, T.K. (2009) Nuclear bodies: random aggregates of sticky proteins or crucibles of macromolecular assembly? *Dev. Cell*. 17, 639–647.

McCabe, D.E., Swain, W.F., Martinell, B.J., Christou, P. (1988) Stable transformation of soybean (*Glycine max*) by particle bombardment. *Biotechnology*, 6, 923–926.

Mizuguchi, M., Obita, T., Kajiyama, A., Kozakai, Y., Nakai, T., Nabeshima, Y. and Okazawa, H. (2016), Allosteric modulation of the binding affinity between PQBP1 and the spliceosomal protein U5-15kD. *FEBS Lett*. 590, 2221–2231.

Morris, D.P., Greenleaf A.L. (2000) The splicing factor, Prp40, binds to the phosphorylated carboxyl-terminal domain of RNA polymerase II. *J. Biol. Chem.* 275, 39935–39943.

Mukhtar, M.S., Carvunis, A.R., Dreze, M., Epple, P., Steinbrenner, J., Moore, J., Tasan, M., Galli, M., Hao, T., Nishimura, M.T., Pevzner, S.J., Donovan, S.E., Ghamsari, L., Santhanam, B., Romero, V., Poulin, M.M., Gebreab, F., Gutierrez, B.J., Tam, S., Monachello, D., Boxem, M., Harbort, C.J., McDonald, N., Gai, L., Chen, H., He, Y., European Union Effectoromics Consortium, Vandenhaute, J., Roth, F.P., Hill, D.E., Ecker, J.R., Vidal, M., Beynon, J., Braun, P., Dangl, J.L. (2011) Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science*. 333, 596-601.

Nabeshima, Y., Mizuguchi, M., Kajiyama, A., Okazawa, H. (2014) Segmental isotope-labeling of the intrinsically disordered protein PQBP1. *FEBS Lett*. 588, 4583–4589.

Nagy, P.D. (2015) Viral sensing of the subcellular environment regulates the assembly of new viral replicase complexes during the course of infection. *J. Virol*. 89, 5196–5199.

Nagy, P.D. (2016) Tombusvirus-Host Interactions: Co-Opted Evolutionarily Conserved Host Factors Take Center Court. *Annu. Rev. Virol*. 3, 491-515.

Nicolaescu, E., Beullens, M., Lesage, B., Keppens, S., Himpens, B. and Bollen, M. (2008) Nature of the nuclear inclusions formed by PQBP1, a protein linked to neurodegenerative polyglutamine diseases. *Eur. J. Cell Biol*. 87, 817–829

Okazawa, H., Rich, T., Chang, A., Lin, X., Waragai, M., Kajikawa, M., Enokido, Y., Komuro, A., Kato, S., Shibata, M., Hatanaka, H., Mouradian, M.M., Sudol, M., Kanazawa, I. (2002) Interaction between mutant ataxin-1 and PQBP-1 affects transcription and cell death. *Neuron*. 34, 701–713.

Okazawa, H., Sudol, M., Rich, T. (2001) PQBP-1 (Np/PQ): a polyglutamine tract-binding and nuclear inclusion-forming protein. *Brain Res. Bull*. *56*, 273–280.

Papantonis, A., Cook, P.R. (2013) Transcription factories: genome organization and gene regulation. *Chem. Rev*. 113, 8683–8705.

Pollard, V.W., Malim, M.H. (1998) The HIV-1 Rev protein. *Annu. Rev. Microbiol*. 52, 491-532.

Qin, J., Barajas, D., Nagy, P.D. (2012) An inhibitory function of WW domain containing host proteins in RNA virus replication. *Virology.* 426, 106–119.

Rojas, M.R., Gilbertson, R.L., Russel, D.R., Maxwell, D.P. (1993) Use of degenerate primers in the polymerase chain reaction to detect whitefly-transmitted geminiviruses. *Plant Dis*. 77, 340–347.

Rojas, M.R., Hagen, C., Lucas, W.J., Gilbertson, R.L. (2005) Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. *Annu. Rev. Phytopathol*. 43, 361–394.

Salah, Z., Alian, A., Aqeilan, R.I. (2012). WW domain-containing proteins: retrospectives and the future. *Front. Biosci*. 17, 331–348.

Sánchez-Velar, N., Udofia, E.B., Yu, Z., Zapp, M.L. (2004) hRIP, a cellular cofactor for Rev function, promotes release of HIV RNAs from the perinuclear region. *Genes Dev*. 18, 23-34.

Shaw, P.J., Brown, J.W. (2004) Plant nuclear bodies. *Curr. Opin. Plant Biol*. 7, 614–620.

Shikata, H., Hanada, K., Ushijima, T., Nakashima, M., Suzuki, Y., Matsushita, T. (2014) Phytochrome controls alternative splicing to mediate light responses in Arabidopsis. *Proc. Natl. Acad. Sci. USA*. 111, 18781–18786.

Sudol, M., Recinos, C.C., Abraczinskas, J., Humbert, J., Farooq, A. (2005) WW or WoW: the WW domains in a union of bliss. *IUBMB Life*. 57, 773–8.

Van Buskirk, E.K., Decker, P.V., Chen, M. (2012) Photobodies in light signaling. *Plant Physiol*. 158, 52–60.

Wang, Q., Moore, M.J., Adelmant, G., Marto, J.A., Silver, P.A. (2013) PQBP1, a factor linked to intellectual disability, affects alternative splicing associated with neurite outgrowth. *Genes Dev*. 27, 615–626.

Waragai, M., Lammers, C.H., Takeuchi, S., Imafuku, I., Udagawa, Y., Kanazawa, I., Kawabata, M., Mouradian, M.M., Okazawa, H. (1999) PQBP-1, a novel polyglutamine tractbinding protein, inhibits transcription activation by Brn-2 and affects cell survival. *Hum. Mol. Genet*. 8, 977–987.

Waragai, M., Junn, E., Kajikawa, M., Takeuchi, S., Kanazawa, I., Shibata, M., Mouradian, M.M., Okazawa, H. (2000) PQBP-1/Npw38, a nuclear protein binding to the polyglutamine tract, interacts with U5-15kD/dim1p via the carboxylterminal domain. *Biochem. Biophys. Res. Commun*. 273, 592–595.

Ward, B.M., Lazarowitz, S.G. (1999) Nuclear Export in Plants: Use of Geminivirus Movement Proteins for a Cell-Based Export Assay. *Plant Cell*. 11, 1267-1276.

Wiermer, M., Palma, K., Zhang, Y., Li, X. (2007) Should I stay or should I go? Nucleocytoplasmic trafficking in plant innate immunity. *Cell Microbiol*. 9, 1880–1890.

Yoh, S.M., Schneider, M., Seifried, J., Soonthornvacharin, S., Akleh, R.E., Olivieri, K.C., De Jesus, P.D., Ruan, C., de Castro, E., Ruiz, P.A., Germanaud, D., des Portes, V., García-Sastre, A., König, R., Chanda, S.K. (2015). PQBP1 is a proximal sensor of the cGAS-dependent innate response to HIV-1. *Cell*.161, 1293–1305.

Zorzatto, C., Machado, J.P.B., Lopes, K.V.G., Nascimento, K.J.T., Pereira, W.A., Brustolini, O.J.B., Reis, P.A.B., Calil, I.P., Deguchi, M., Sachetto-Martins, G., Gouveia, B.C., Loriato, V.A.P., Silva, M.A.C., Silva, F.F., Santos, A.A., Chory, J., Fontes, E.P.B. (2015) NIK1-mediated translation suppression functions as a plant antiviral immunity mechanism. *Nature*. 520, 679-682

Supplementary Information

Table S1. Differential expression of AT2G41020 (AtWWP1) in different genotypes by RNA sequencing

	Log2FC	Adjusted p-value
AtWWP1 4 – Col 0	2.86399	7.81E-62
AtWWP1 1 – Col 0	0.60823	0.00179
Knockout – Col 0	-3.97319	3.95E-75

Table S2: List of pimers used for cloning, diagnose, RT-PCR and qPCR

Primer	Annealing gene	Sequence
At2G41020-Fwd	At2G41020/AtWWP1	AAAAAGCAGGCTTCACAATGGGAGAAGAGCTGCA
At2G41020-ST-Rvs	At2G41020/AtWWP1	AGAAAGCTGGGTCTCAATCTGCGTCTCCAAGACC
At2G41020-NS-Rvs	At2G41020/AtWWP1	AGAAAGCTGGGTCATCTGCGTCTCCAAGACC
NIG_4263-Fwd	At4G13350/NIG	AAAAAGCAGGCTTCACAATGGCGGGTCGAGTTAA
NIG_4264-NS-Rvs	At4G13350/NIG	AGAAAGCTGGGTCTTACCCAAATGGGTTTCCTCC
NIG_4265-ST-Rvs	At4G13350/NIG	AGAAAGCTGGGTCCCCAAATGGGTTTCCTCCTGA
CSN5A-Fwd	At1G22920/CSN5A	AAAAAGCAGGCTTCACAATGGAAGGTTCCTCGTC
CSN5A-ST-Rvs	At1G22920/CSN5A	AGAAAGCTGGGTCTCACGATGTAATCATGGGCTC
CSN5A-NS-Rvs	At1G22920/CSN5A	AGAAAGCTGGGTCCGATGTAATCATGGGCTC

WWP1(192)-Rvs	At2G41020/AtWWP1-192aa	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCTTGCATTAGCCTGAAAAGG
WWP1(271)-Rvs	At2G41020/AtWWP1-271aa	GGGGACCACTTTGTACAAGAAAGCTGGGTCAGAAGCTGGAGGTTCCC
WWP1(193)-Fwd	At2G41020/AtWWP1-193aa	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGACCTTACCATTAGG
WWP1(272)-Fwd	At2G41020/AtWWP1-272aa	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTTACAGAAGCCCGCTGC
AtWWP1W198A	At2G41020/AtWWP1 W198A	GCAAGCACCTTACCATTAGGAGCTGTAGATGCAAAAGATCCTGCC
AtWWP1W221A	At2G41020/AtWWP1 W221A	CAGCACACTGGAACATGCCAGGCTGAAAGGCCTGTTGAGCTTTC
AtWWP1W243A	At2G41020/AtWWP1 W243A	CCTGTACTTTCTAAAGAAGAGGCTATTGAAACATTTGATGAAGC
AtWWP1W266A	At2G41020/AtWWP1 W266A	CAAGGACACATGTGTCTCAGGCTGAACCTCCAGCTTCTTTACAG
DEST32-Fwd	pDEST32 vector	AACCGAAGTGCGCCAAGTGTCTG
DEST22-Fwd	pDEST22 vector	TATAACGCGTTTGGAATCACT
DEST22-Rvs	pDEST32 / pDEST22 vector	AGCCGACAACCTTGATTGGAGAC
PBL1v2040	begomovirus-specific primers (Rojas et al., 1993)	GCCTCTGCAGCARTGRTCKATCTTCATACA
PCRC1		CTAGCTGCAGCATATTTACRARWATGCCA

	begomovirus-specific primers (Rojas et al., 1993)	
attB1_2942	Gateway AttB1site sequence	GGGGACAAGTTTGTACAAAAAAGCAGGCT
attB1_2943	Gateway AttB1 site sequence	GGGGACCACTTTGTACAAGAAAGCTGGGT
pDONR201/ 207_3397-Fwd	pDONR207	TCGCGTTAACGCTAGCATGGATC
pDONR201/ 207_3398 (Rvs)	pDONR201	TGTAACATCAGAGATTTTGAGACAC
35S_MC36 (Fwd)	35S promoter	TCCTTCGCAAGACCCTTCCTC
GFP_4799 (Rvs)	Green Fluorescent Protein (GFP)	CGCCCTCGCCCTCGCCGGACAC
qRT_CaLCuV-Fwd	CaLCuV DNA-B	GGGCCTGGGCCTGTTAGT
qRT_CaLCuV-Rvs	CaLCuV DNA-B	ACGGAAGATGGGAGAGGAAGA
qRT_18SRNA-Fwd	Arabidopsis 18SRNA	TTTGCGCGCCTGCTGCC
qRT_18SRNAV-Rvs	Arabidopsis 18SRNA	TGTGCTGGCGACGCATCATT
pWWP1_2KB-FWD	At2G41020/AtWWP1 promoter	GGGGACAACTTTGTATAGAAAAGTTGTC GATTTGCGTATTCTATTT
pWWP1_2KB-RVS	At2G41020/AtWWP1 promoter	GGGGACTGCTTTTTTGTACAAACTTGC TATTTAGTTCAGCGTCAACCT
pWWP1_1KB-FWD	At2G41020/AtWWP1	GAATTCACGGTCACACCGTTTATTACG

	promoter	
pWWP1_1KB-RVS	At2G41020/AtWWP1 promoter	AGATCTTATTTAGTTCAGCGTCAACCTC

Figures legends

Figure 1. AtWWP1 associates with NIG in the nucleus of plant cells
(A) Yeast two-hybrid assay of AtWWP1 and NIG interaction. AtWWP1 and NIG proline-rich domain (PRNIG) were expressed in yeast fused to either the activation domain (AD) and the binding domain (BD) of GAL4. The interactions between the tested proteins were analyzed by monitoring His prototrophy on seletive medium supplemented with 3AT. The full-length NIG fused to AD, which displays transactivation activity in yeast, was used as positive control.
(B) Transactivation of the LACZ reporter gene. The interactions were confirmed by measuring the activity of the β-galactosidase reporter enzyme activity The transactivation capacity of NIG was monitored as a positive control. Asterisks indicate statistically significant differences comparing to the negative controls (t-test, $p \leq 0,05$, n=3).
(C) AtWWP1 interacts with NIG *in planta*. Protein extracts from *N. benthamiana* leaves transiently expressing AtWWP1-GFP and NIG-HA fusions were used for co-imunoprecipitation assays. The CoIP was performed using an anti-GFP antibody. Inputs and IP controls show AtWWP1-GFP and NIG-HA protein levels. Anti-haemagglutinin (HA) antibody was used to detect NIG-HA from the immunoprecipitated complex. RPL10A-GFP was used as an unrelated protein.
(D) BIFC assay showing AtWWP1 and NIG interaction in the nucleus of *N. benthamiana* cells. Constructs expressing AtWWP1 and NIG fused to the YFP C-terminus (cYFP) or N-terminus (nYFP) were observed under the confocal microscope 3 days after infiltration. DIC, differential interference contrast.

Figure 2. AtWWP1 forms nuclear bodies
(A) Nuclear distribution of AtWWP1 in *N. benthamiana* leaves infiltrated with *A. tumefaciens* carrying AtWWP1-GFP constructs under 35S constitutive promoter. AtWWP1-GFP was imaged by confocal microscope 36 hours after infiltration. The nuclear inclusion pattern was observed in 89.51% ± 0.07 cells. Approximately 120 cells were examined.
(B) Confocal fluorescence image of transiently expressed AtWWP1-YFP under its native promoter control.
(C) Confocal image of Arabidopsis transgenic root cells stably transformed with 35::AtWWP1-GFP. Scale bars, 10 μm.

Figure 3. AtWWP1 co-localizes with NIG in nuclear bodies
(A) Cytosolic and perinuclear distribution of NIG fused either to C-terminal mCherry tag and N-terminus YFP tag. Confocal image shows subcellular localization of NIG-mCherry and YFP-NIG transiently expressed in *N. bethamiana* leaf cells.
(B) Confocal fluorescence image of Arabidopsis root cells stably transformed with 35S::NIG-GFP.
(C) Co-localization of NIG with AtWWP1 in nuclear bodies. Confocal image of subcellular localization of NIG-mCherry transiently co-expressed with AtWWP1-GFP in *N. bethamiana* leaf cells
(D) Confocal fluorescence image of Arabidopsis root cells stably co-transformed with 35S::NIG-mCherry and 35S::AtWWP1-GFP. The arrow denotes nuclear co-localization of AtWWP1 and NIG. Scale bars, 20 μm.

Figure 4. AtWWP1 confers tolerance to begomovirus
(A) CaLCuV infection-associated symptoms in wild-type, *AtWWP1*-ovexpressing and knockout lines at 21 dpi. Plants were infected with CaLCuV by biolistic delivery and the

course of infection was monitored by PCR amplification of viral DNA. The figure shows representative samples of Col-0, AtWWP1-GFP_1, AtWWP1-GFP_4 and *atwwp1* plants.
(B) Course of infection in Col-0, AtWWP1-GFP_1, AtWWP1-GFP_4 and *atwwp1* infected plants.
(C) Absolute quantitation of CaLCuV genomic units in infected lines at 14 dpi. Error bars indicate 95% confidence intervals based on bootstrap resampling replicates of three independent experiments.

Figure 5. Stability of AtWWP1 nuclear bodies upon begomovirus infection.
Tobbaco leaves infected with TGMV were co-infiltrated with 35S::AtWWP1-GFP and 35S::NSP-mCherry constructions. Images were assayed by cofocal microscopy 3 days after infiltration. Arrow denotes nucleus of infected cells expressing AtWWP1-GFP in a diffused pattern.Scale bars, 20 μm.

Figure 6. WW domains are essential for AtWWP1 nuclear body formation
(A) AtWWP1$^{1\text{-}193}$-GFP, AtWWP1$^{1\text{-}271}$-GFP, AtWWP1$^{193\text{-}271}$-GFP, AtWWP1$^{193\text{-}361}$-GFP, AtWWP1$^{193\text{-}463}$-GFP, AtWWP1$^{272\text{-}463}$-GFP and AtWWP1$^{362\text{-}463}$-GFP fusion proteins harboring different fragments of AtWWP1 were expressed in *N. benthamiana* leaves via *A. tumefaciens*–mediated expression. Confocal images represent the predominant pattern, as indicated by percentage values followed by standard deviation. Scale bars, 10 μm.
(B) Western blot analysis of protein levels from AtWWP1 fragments detected by Anti-GFP antibody.

Figure 7. AtWWP1 mutants are defective in nuclear body-formation capacity
(A) Replacement of tryptophan (W) residues with alanine (A) in the AtWWP1-GFP-fused protein. Confocal images represent the percentage of assayed cells displaying the indicated pattern. In all experiments more than 50 cells were examined. Scale bars, 10 μm
(B) Western blot analysis of protein levels from AtWWP1 mutant forms detected by anti-GFP antibody
(C)Yeast two-hybrid assay of PRNIG and AtWWP1^{W198AW243AW266A} (AWAA) mutant interaction. AD-AtWWP1/AWAA and BD-PRNIG recombinant protein were expressed in yeast and the interactions between the tested proteins were analyzed by monitoring His prototrophy on seletive medium.
(D) Co-localization of NIG with AtWWP1/AWAA mutant in *N. benthamiana* cells. Confocal images were assayed 3 days after inoculation. Scale bars, 10 μm.

Figure 8. AtWWP1 interacts with CSN5A
(A)Yeast two-hybrid assay of AtWWP1 and CSN5A interaction. AD-AtWWP1 and BD-CSN5A recombinant protein were expressed in yeast and the interactions between the tested proteins were analyzed by monitoring His prototrophy on seletive medium supplemented with 10mM3AT.
(B) Co-immunoprecipitation (CoIP) assay of AtWWP1 and CSN5A from *N. benthamiana* leaves. Protein complexes were isolated using anti-GFP magnetic beads, separated by SDS–PAGE, and immunoblotted with the indicated antibody. Anti-haemagglutinin (HA) antibody was used to detect AtWWP1-HA from the immunoprecipitated complex.
(C) AtWWP1 and CSN5A interact in the nucleus of *N. benthamiana* cells. Constructs expressing AtWWP1 and CSN5A fused to the YFP C-terminus (cYFP) or N-terminus (nYFP) were observed under the confocal microscope 3 days after infiltration. YFP fluorescence was observed in cells co-infiltrated with constructs corresponding to nYFP-CSN5A and cYFP-AtWWP1. Scale bars, 10 μm. DIC, differential interference contrast.

(D) Subnuclear co-localization of AtWWP1-GFP and CSN5A-mCherry in the nucleus of *N. benthamiana* transfected cells. Scale bars, 10 μm.

Figure 9. AtWWP1 confers tolerance to begomovirus by sequestering NIG into nuclear bodies

Begomoviruses replicate their circular, single-stranded DNA genomes via double-stranded DNA intermediates in nuclei of infected cells. The newly synthesized single-stranded DNA (ssDNA) is translocated across the nuclear envelope trough association with viral NSP. The nuclear exportation of NSP-DNA complex is facilitated by NIG, a perinuclear localized host factor that acts as a cofactor for NSP translocation function. NIG can dynamically changes its subcellular localization, trafficking from cytoplasm to nucleus, which may be positively explored by begomoviruses, during nuclear shuttling of viral genome. AtWWP1, a NIG partner, forms immune nuclear bodies that may sequester host factors targeted by viral proteins during infection. AtWWP1 interacts with NIG and confines it into nuclear bodies, preventing its association with NSP. Viral DNA may also interact with AtWWP1-NBs as a counter defensive measure. Additionally, AtWWP1 integrates a plant immune hub converging to CSN5A, which may help to modulate immune response against viral pathogens. Continuous arrows indicate proved interactions and dashed arrows denote putative interactions.

Supplementary Figures Legends

Figure S1. *In silico* analysis of AtWWP1 protein

(A) Phylogenetic tree representing the predicted relationship between AtWWP1 (AT2G41020) and WW domain-containing proteins in agronomic crops and Arabidopsis. The AtWWP1 clade is highlighted in red.

(B) Phylogenetic tree of WW-domain-containing proteins from Arabidopsis. The AtWWP1 clade is highlighted in grey. The amino acid sequences containing WW domain were aligned using MUSCLE. Phylogenetic trees (A) and (B) were constructed using Bayesian inference performed with MrBayes v3.2.2 with the mixed amino acid substitution model (Jones).

(C) Multiple alignment of AtWWP1 with Prp40 proteins from Arabidopsis. The WW domain regions are highlighted in grey.

(D) Sequence alignment of AtWWP1 and human PQBP1. A C-terminus conserved motif is indicated in dark grey and a WW domain in light gray. Both alignments, (C) and (D), were carried out with CLUSTAL OMEGA. Identical amino acids are indicated with asterisks, highly conserved residues with (**:**) and weakly conserved (**.**)

Figure S2. Tissue-specific expression of the AtWWP1 promoter

GUS reporter gene expression was histochemically monitored in 2-week-old seedling leaves and roots from transgenic lines harboring a β-glucuronidase (GUS) reporter gene expressed from the AtWWP1 and NIG promoters. The figure shows histochemical analysis of GUS activity of three independent lines.

Figure S3. AtWWP1 transcript levels and protein accumulation in transgenic lines

(A) Accumulation of AtWWP1 transcript levels in wild type and transgenic lines, monitored by qRT-PCR and normalized to actin. Error bars, 95% confidence intervals (n=3) based on bootstrap resampling replicates of three independent experiments.

(B) Accumulation of AtWWP1-GFP protein levels in Col-0, AtWWP1 ovexpressing lines and atwwp1 plants. Protein extracts were immunoblotted with anti-GFP antibodies.

(C) Schematic representation of T-DNA insertion site in *atwwp1* (Salk_073780). Triangles

show the positions of the T-DNA insertion in AtWWP1 sequence. The thick lines depict introns, green boxes mean exons. Grey boxes represent the 5′- and 3′-UTRs.

Figure S4. General downregulation of differentially expressed genes related to immune response in AtWWP1ox and atwwp1 mutant lines

Schematic representation of Plant-Pathogen Pathway view. Up- and down-regulated genes in Arabidopsis *AtWWP1*-overexpressing line-4 and *atwwp1* mutant are shown in green and red, respectively.

Figure S5. AtWWP1 forms dimmers through its WW domain

(A) Yeast two-hybrid assay of AtWWP1 fused to GAL4 domains. AtWWP1-AD and AtWWP1-BD were expressed in yeast and assayed for interaction on selective medium supplemented with 3AT.

(B) Yeast two-hybrid analysis of association between AtWWP1 and its truncated forms. $AtWWP1^{1-193}$, $AtWWP1^{1-271}$, $AtWWP1^{193-463}$ and $AtWWP1^{272-463}$ fragments were fused to GAL4 BD domains and co-expressed in yeast with AD-AtWWP1 construction. The interactions between the tested proteins were analyzed on selective medium supplemented with 3AT.

(C) *In vivo* AtWWP1 self-interaction. *N. benthamiana* leaves were co-infiltrated with the constructions cYFP-AtWWP1, nYFP-AtWWP1 or empty vectors and examined using the confocal microscope 3 day after infiltration. Scale bars, 10 μm.

(D) Interaction analysis between AtWWP1 and AtWWP1-truncated forms. $AtWWP1^{1-193}$, $AtWWP1^{1-271}$, $AtWWP1^{193-463}$ and $AtWWP1^{272-463}$ truncated forms were fused to YFP N-terminal (nYFP) and co-expressed in *N. benthamiana* leaves with cYFP-AtWWP1 or empty vector. The images were acquired 3 days after infiltration using a confocal microscope. Scale bars, 10 μm.

Figure S6. Begomovirus-Interacting Immune hub

Schematic representation of previously identified interactions involving begomovirus proteins (red) and host proteins (green) converging to CSN5A (blue), the central component of the immune hub. (Carvallho *et al*., 2008; Fontes *et al*.,2004; Lozano-Durán *et al*., 2011; Mukhtar *et al*., 2011)

Figures

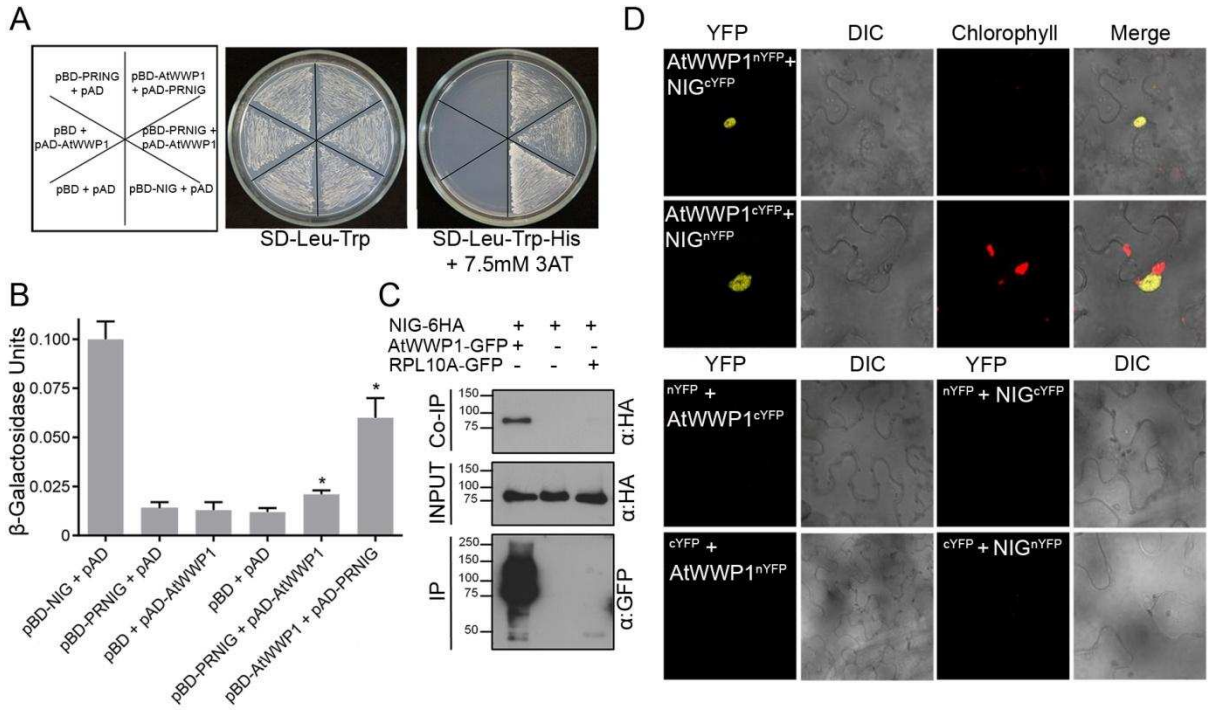

Figure 1. AtWWP1 associates with NIG in the nucleus of plant cells

(A) Yeast two-hybrid assay of AtWWP1 and NIG interaction. AtWWP1 and NIG-proline-Rich domain (PRNIG) were expressed in yeast fused to either the activation domain (AD) and the binding domain (BD) of GAL4. The interactions between the tested proteins were analyzed by monitoring His prototrophy on seletive medium supplemented with 3AT. The full-length NIG fused to AD, which displays transactivation activity in yeast, was used as positive control.

(B) Transactivation of the LACZ reporter gene. The interactions were confirmed by measuring the activity of the β-galactosidase reporter enzyme activity The transactivation capacity of NIG was monitored as a positive control. Asterisks indicate statistically significant differences comparing to the negative controls (t-test, $p \leq 0{,}05$, n=3).

(C) AtWWP1 interacts with NIG *in planta*. Protein extracts from *N. benthamiana* leaves transiently expressing AtWWP1-GFP and NIG-HA fusions were used for co-imunoprecipitation assays. The CoIP was performed using an anti-GFP antibody. Inputs and IP controls show AtWWP1-GFP and NIG-HA protein levels. Anti-haemagglutinin (HA) antibody was used to detect NIG-HA from the immunoprecipitated complex. RPL10A-GFP was used as an unrelated protein.

(D) BIFC assay showing AtWWP1 and NIG interaction in the nucleus of *N. benthamiana* cells. Constructs expressing AtWWP1 and NIG fused to the YFP C-terminus (cYFP) or N-terminus (nYFP) were observed under the confocal microscope 3 days after infiltration. DIC, differential interference contrast.

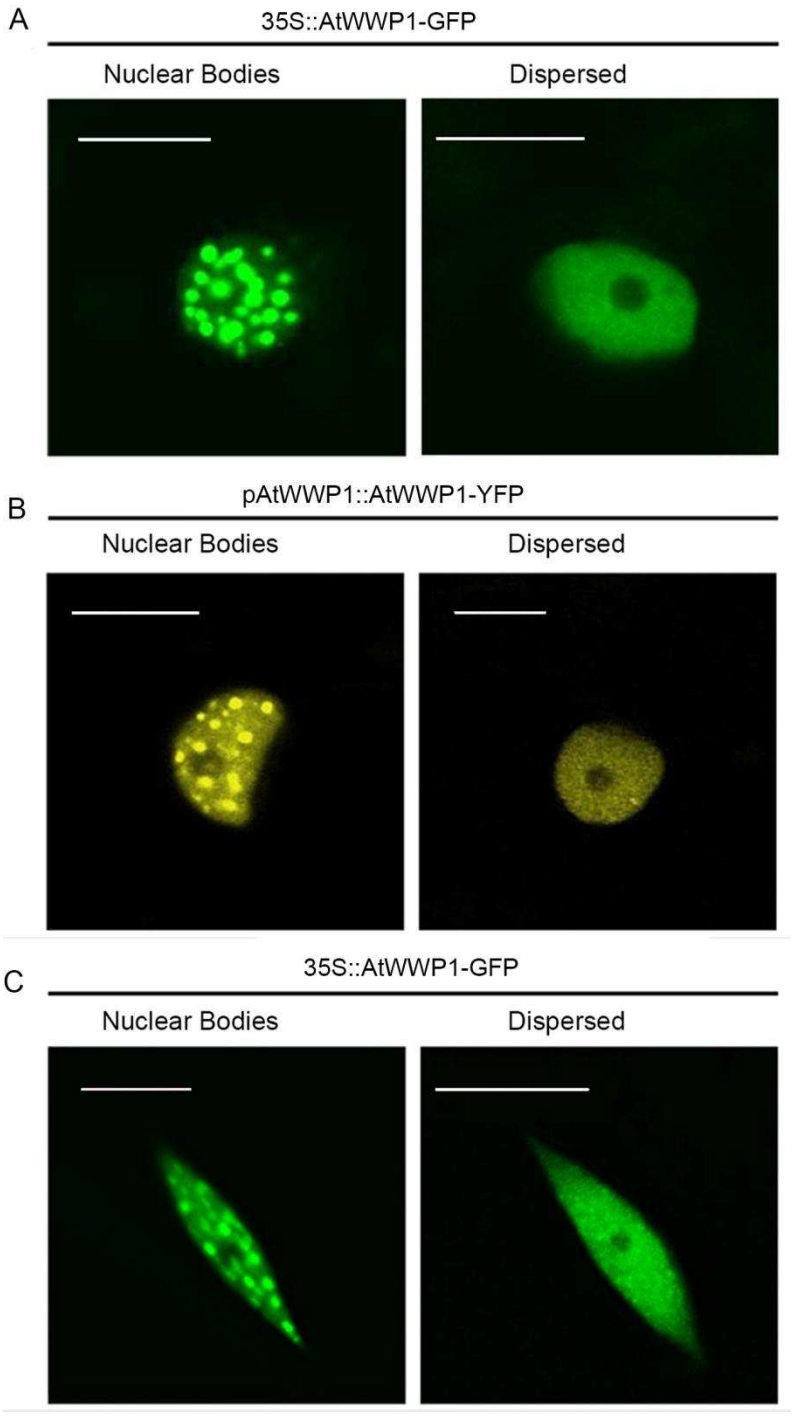

Figure 2. AtWWP1 forms nuclear bodies

(A) Nuclear distribution of AtWWP1 in *N. benthamiana* leaves infiltrated with *A. tumefaciens* carrying AtWWP1-GFP constructs under 35S constitutive promoter. AtWWP1-GFP was imaged by confocal microscope 36 hours after infiltration. The nuclear inclusions pattern were observed in 89.51% ± 0.07 cells. Approximately 120 cells were examined.

(B) Confocal fluorescence image of transiently expressed AtWWP1-YFP under its native promoter control.

(C) Confocal image of Arabidopsis transgenic root cells stably transformed with 35::AtWWP1-GFP. Scale bars, 10 μm.

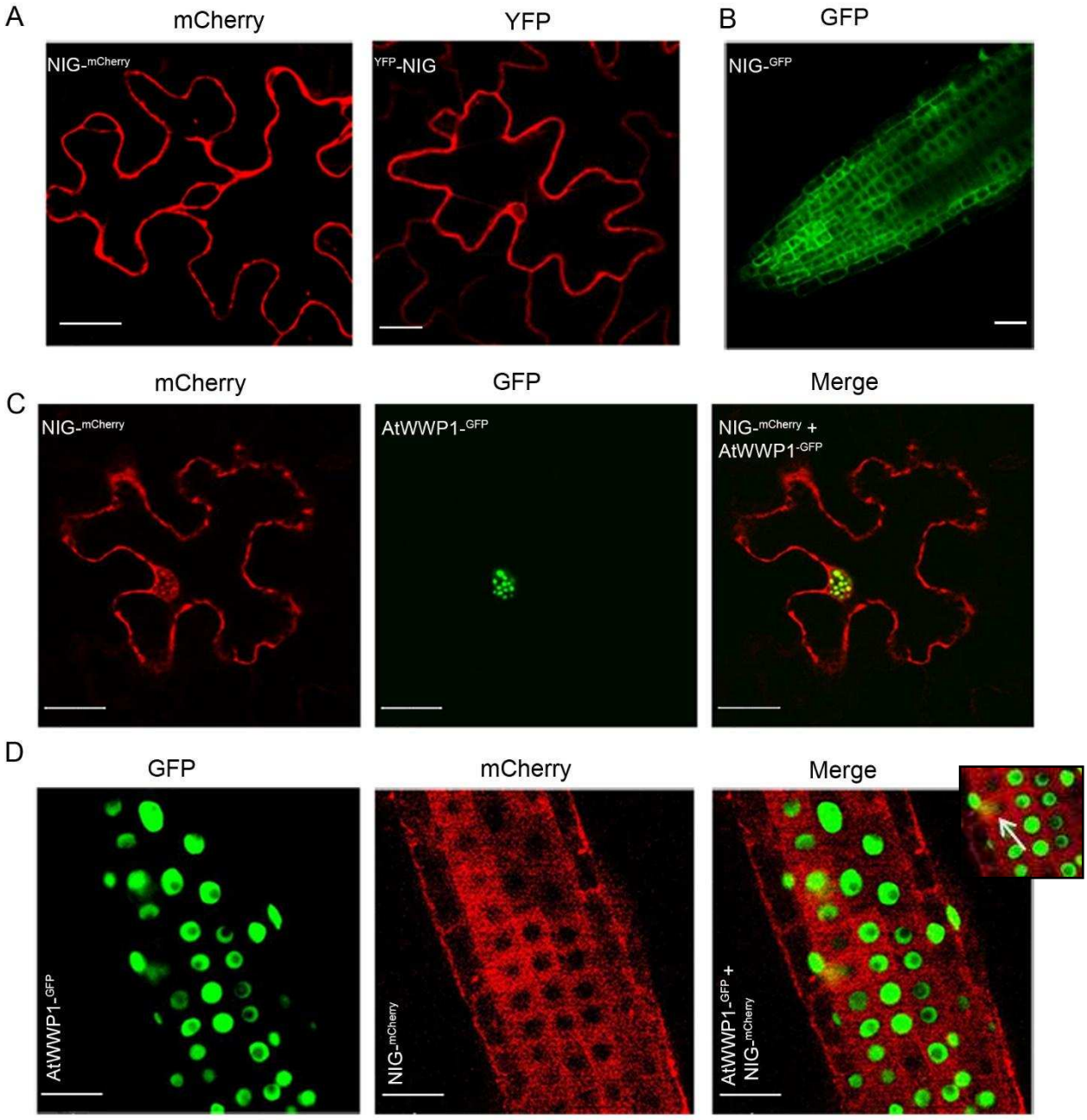

Figure 3. AtWWP1 co-localizes with NIG in nuclear bodies

(A) Cytosolic and perinuclear distribution of NIG fused either to C-terminal mCherry tag and N-terminus YFP tag. Confocal image shows subcellular localization of NIG-mCherry and YFP-NIG transiently expressed in *N. bethamiana* leaf cells.

(B) Confocal fluorescence image of Arabidopsis root cells stably transformed with 35S::NIG-GFP.

(C) Co-localization of NIG with AtWWP1 in nuclear bodies. Confocal image of subcellular localization of NIG-mCherry transiently co-expressed with AtWWP1-GFP in *N. bethamiana* leaf cells

(D) Confocal fluorescence image of Arabidopsis root cells stably co-transformed with 35S::NIG-mCherry and 35S::AtWWP1-GFP. The arrow denotes nuclear co-localization of AtWWP1 and NIG. Scale bars, 20 μm.

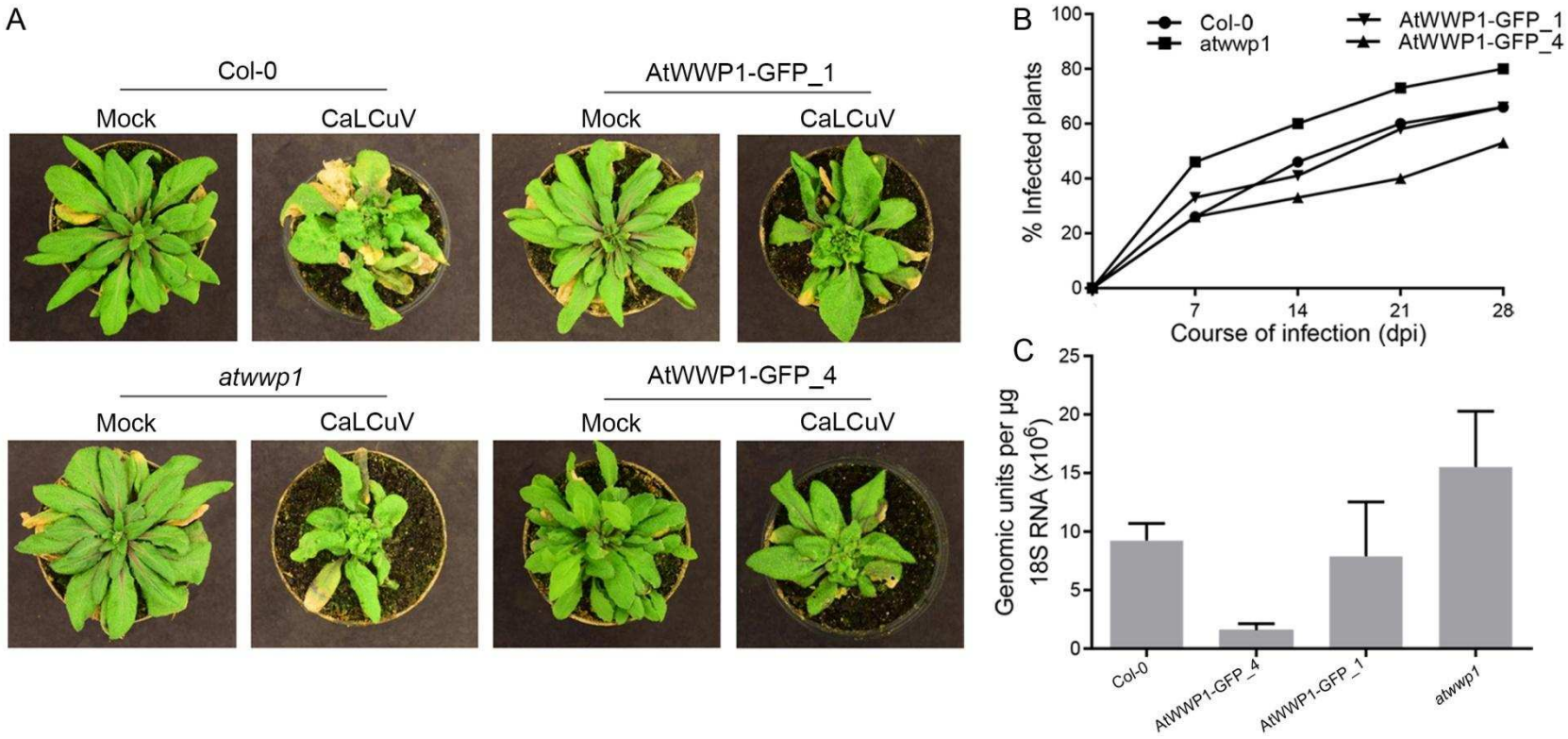

Figure 4. AtWWP1 confers tolerance to begomovirus

(A) CaLCuV infection-associated symptoms in wild-type, *AtWWP1*-ovexpressing and knockout lines at 21 dpi. Plants were infected with CaLCuV by biolistic delivery and the course of infection was monitored by PCR amplification of viral DNA. The figure shows representative samples of Col-0, AtWWP1-GFP_1, AtWWP1-GFP_4 and *atwwp1* plants.

(B) Course of infection in Col-0, AtWWP1-GFP_1, AtWWP1-GFP_4 and *atwwp1* infected plants.

(C) Absolute quantitation of CaLCuV genomic units in infected lines at 14 dpi. Error bars indicate 95% confidence intervals based on bootstrap resampling replicates of three independent experiments.

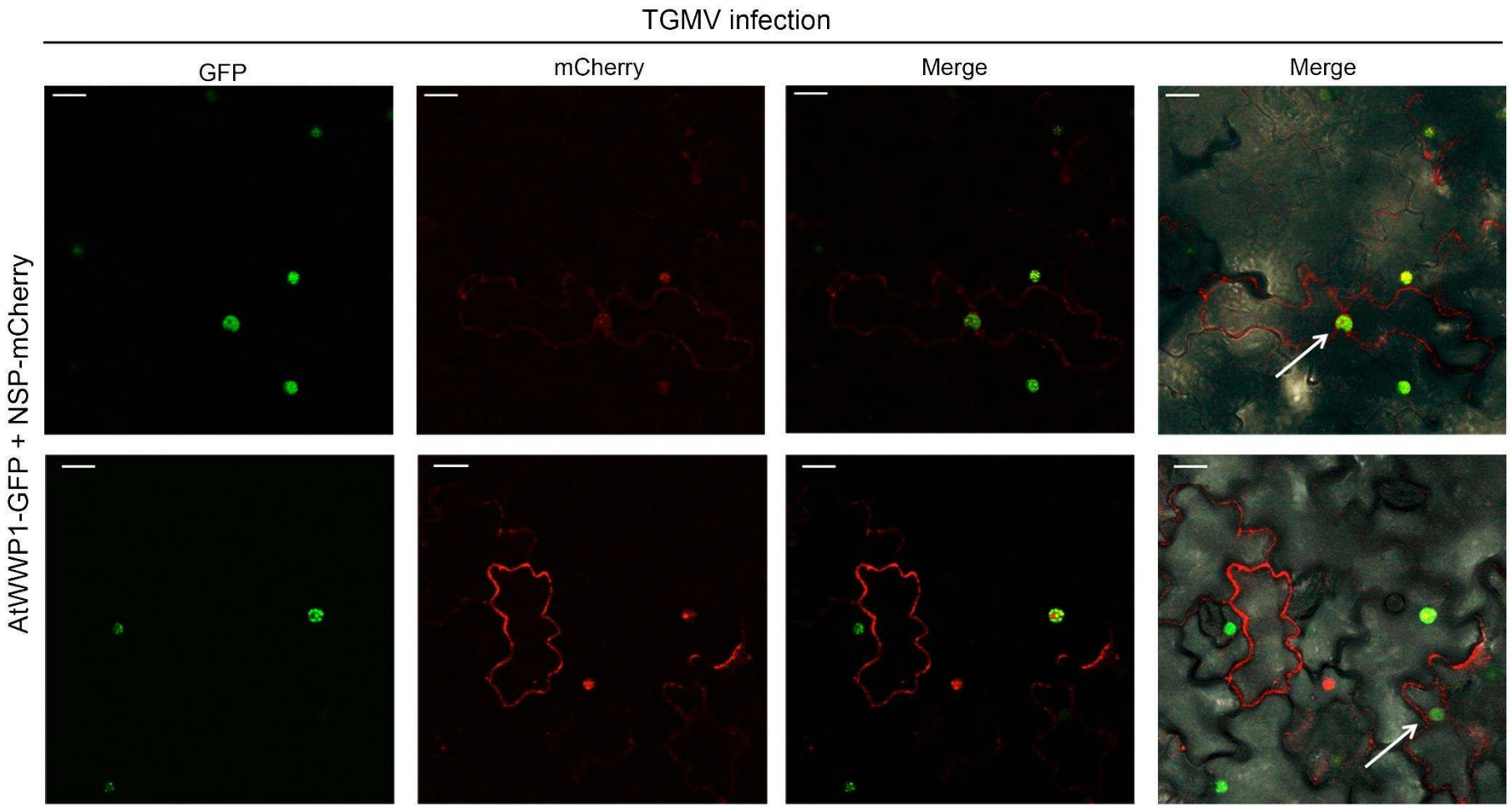

Figure 5. Stability of AtWWP1 nuclear bodies upon begomovirus infection.

Tobbaco leaves infected with TGMV were co-infiltrated with 35S::AtWWP1-GFP and 35S::NSP-mCherry constructions. Images were assayed by cofocal microscopy 3 days after infiltration. Arrow denotes nucleus of infected cells expressing AtWWP1-GFP in a diffused pattern.Scale bars, 20 μm.

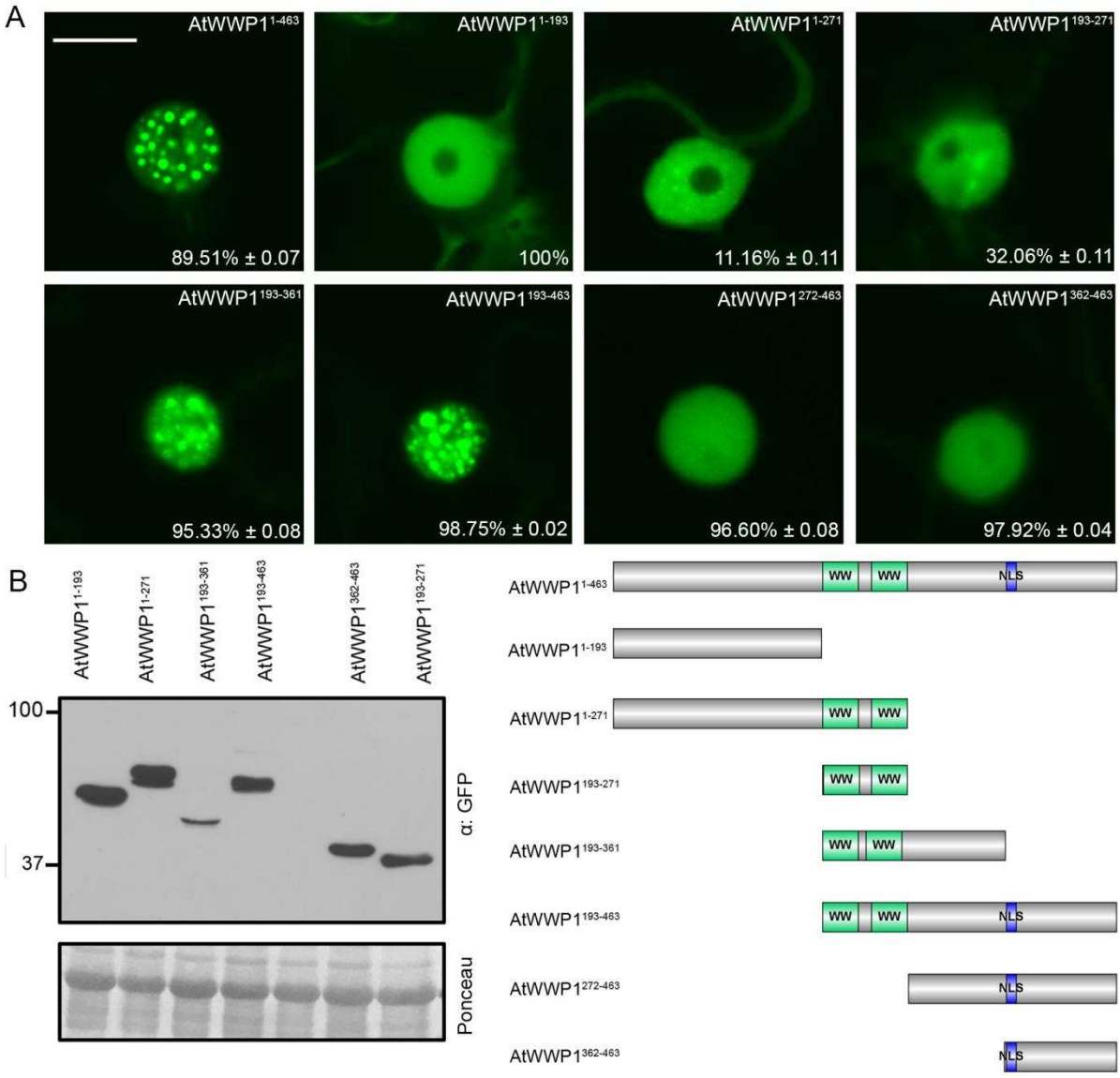

Figure 6. WW domains are essential for AtWWP1 nuclear body formation
(A) AtWWP1$^{1-193}$-GFP, AtWWP1$^{1-271}$-GFP, AtWWP1$^{193-271}$-GFP, AtWWP1$^{193-361}$-GFP, AtWWP1$^{193-463}$-GFP, AtWWP1$^{272-463}$-GFP and AtWWP1$^{362-463}$-GFP fusion proteins harboring different fragments of AtWWP1 were expressed in *N. benthamiana* leaves via *A. tumefaciens*–mediated expression. Confocal images represent the predominant pattern, as indicated by percentage values followed by standard deviation. Scale bars, 10 µm.
(B) Western blot analysis of protein levels from AtWWP1 fragments detected by Anti-GFP antibody.

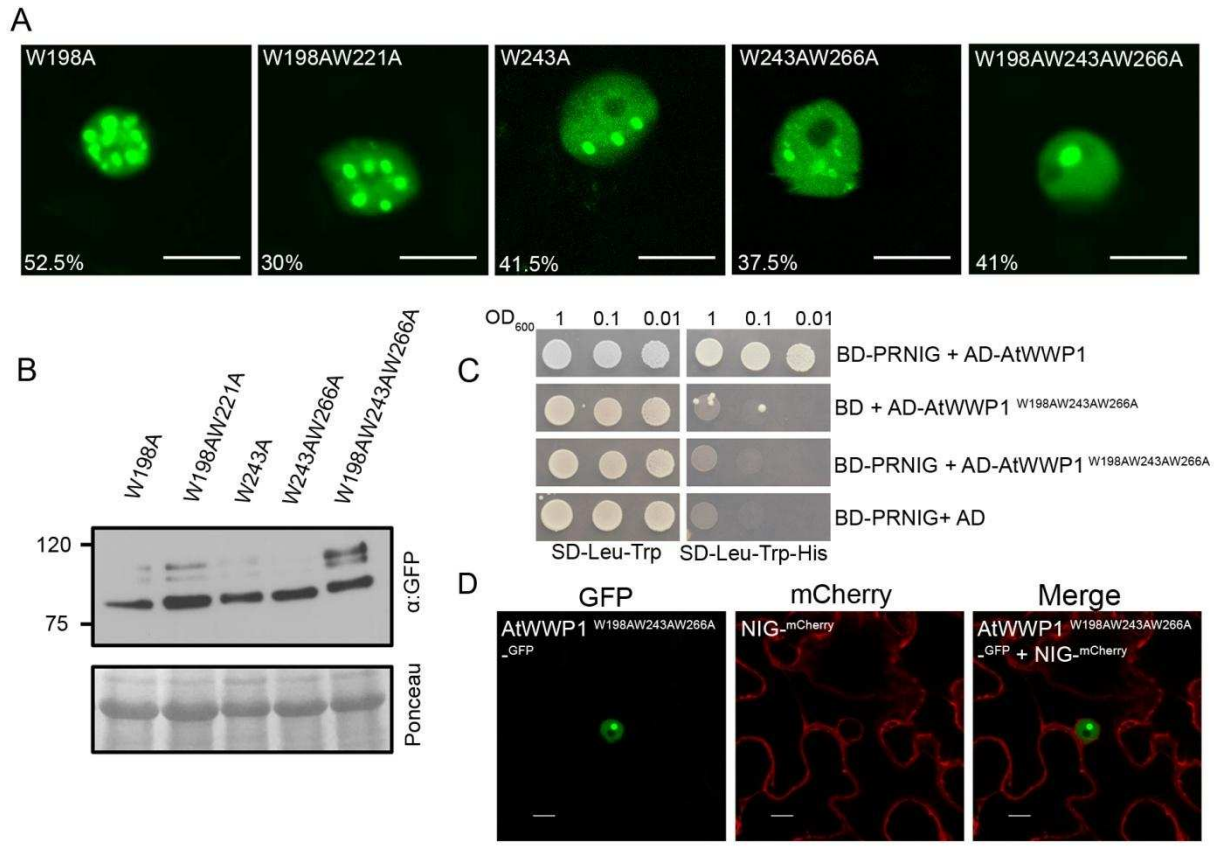

Figure 7. AtWWP1 mutants are defective in nuclear body-formation capacity
(A) Replacement of tryptophan (W) residues with alanine (A) in the AtWWP1-GFP-fused protein. Confocal images represent the percentage of assayed cells displaying the indicated pattern. In all experiments more than 50 cells were examined. Scale bars, 10 μm
(B) Western blot analysis of protein levels from AtWWP1 mutant forms detected by anti-GFP antibody
(C) Yeast two-hybrid assay of PRNIG and AtWWP1^{W198AW243AW266A} (AWAA) mutant interaction. AD-AtWWP1/AWAA and BD-PRNIG recombinant protein were expressed in yeast and the interactions between the tested proteins were analyzed by monitoring His prototrophy on seletive medium.
(D) Co-localization of NIG with AtWWP1/AWAA mutant in *N. benthamiana* cells. Confocal images were assayed 3 days after inoculation. Scale bars, 10 μm.

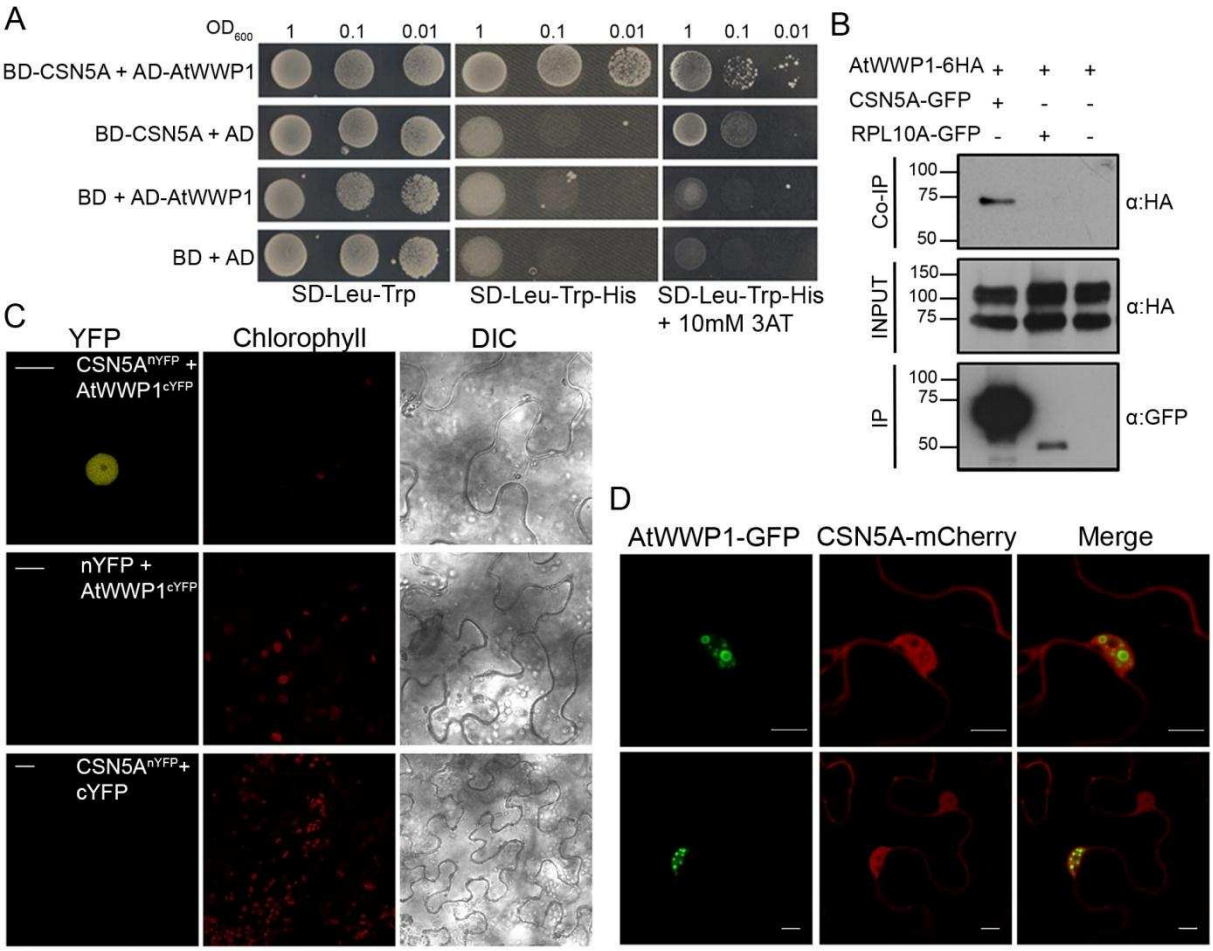

Figure 8. AtWWP1 interacts with CSN5A

(A)Yeast two-hybrid assay of AtWWP1 and CSN5A interaction. AD-AtWWP1 and BD-CSN5A recombinant protein were expressed in yeast and the interactions between the tested proteins were analyzed by monitoring His prototrophy on seletive medium supplemented with 3AT.

(B) Co-immunoprecipitation (CoIP) assay of AtWWP1 and CSN5A from *N. benthamiana* leaves. Protein complexes were isolated using anti-GFP magnetic beads, separated by SDS–PAGE, and immunoblotted with the indicated antibody. Anti-haemagglutinin (HA) antibody was used to detect AtWWP1-HA from the immunoprecipitated complex.

(C) AtWWP1 and NIG interact in the nucleus of *N. benthamiana* cells. Constructs expressing AtWWP1 and NIG fused to the YFP C-terminus (cYFP) or N-terminus (nYFP) were observed under the confocal microscope 3 days after infiltration. YFP fluorescence was observed in cells co-infiltrated with constructs corresponding to nYFP-CSN5A and cYFP-AtWWP1. Scale bars, 10 μm. DIC, differential interference contrast.

(D) Subnuclear co-localization of AtWWP1-GFP and CSN5A-mCherry in the nucleus of *N. benthamiana* transfected cells. Scale bars, 10 μm.

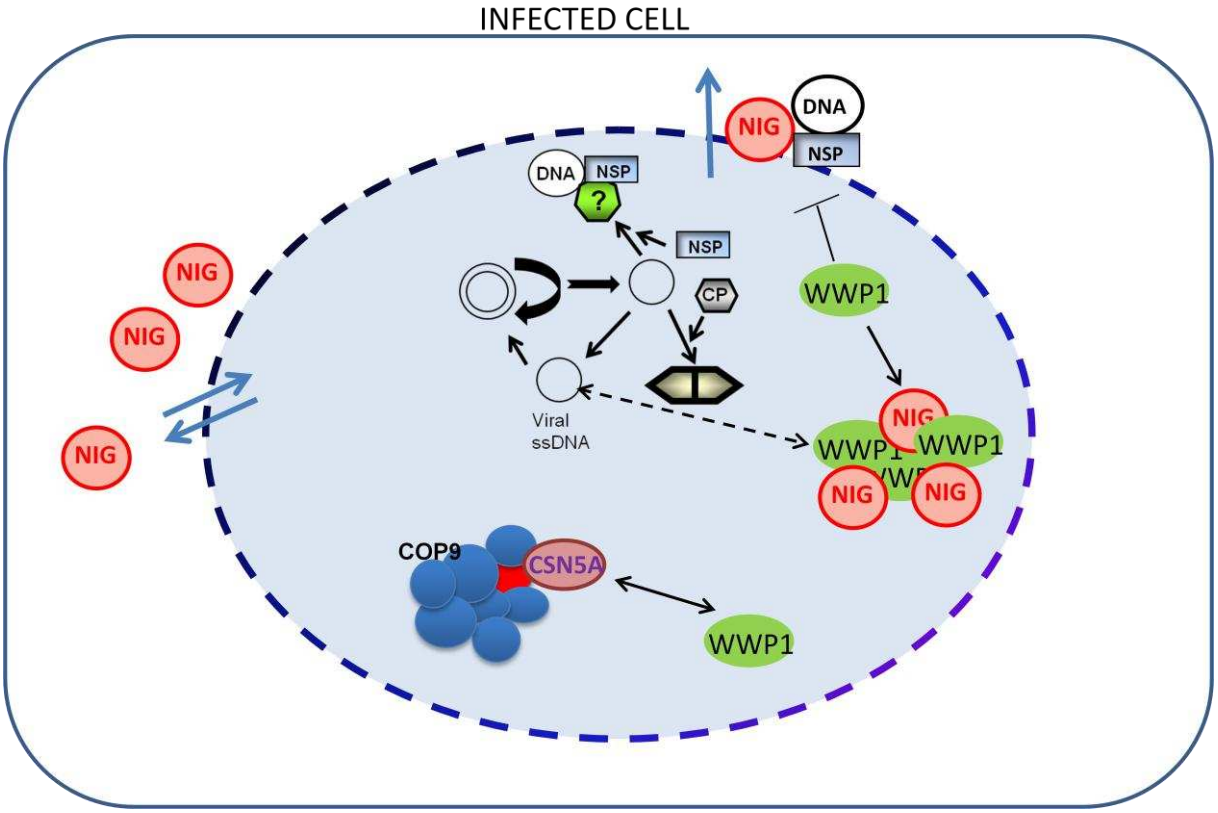

Figure 9. AtWWP1 confers tolerance to begomovirus by sequestering NIG into nuclear bodies

Begomoviruses replicate their circular, single-stranded DNA genomes via double-stranded DNA intermediates in nuclei of infected cells. The newly synthesized single-stranded DNA (ssDNA) is translocated across the nuclear envelope trough association with viral NSP. The nuclear exportation of NSP-DNA complex is facilitated by NIG, a perinuclear localized host factor that acts as a cofactor for NSP translocation function. NIG can dynamically changes its subcellular localization, trafficking from cytoplasm to nucleus, which may be positively explored by begomoviruses, during nuclear shuttling of viral genome. AtWWP1, a NIG partner, forms immune nuclear bodies that may sequester host factors targeted by viral proteins during infection. AtWWP1 interacts with NIG and confines it into nuclear bodies, preventing its association with NSP. Viral DNA may also interact with AtWWP1-NBs as a counter defensive measure. Additionally, AtWWP1 integrates a plant immune hub converging to CSN5A, which may help to modulate immune response against viral pathogens. Continuous arrows indicate proved interactions and dashed arrows denote putative interactions.

Supplementary Figures

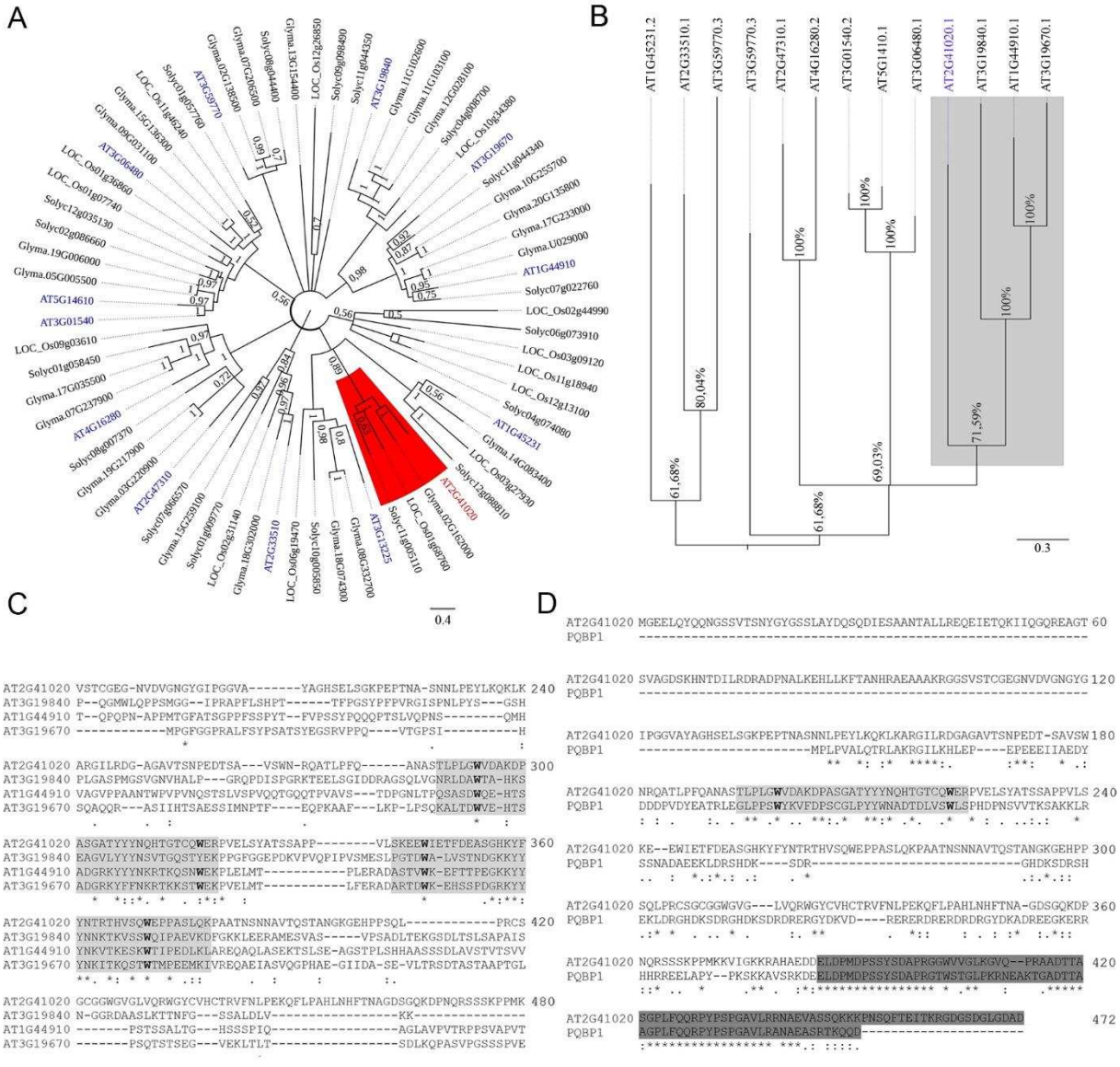

Figure S1. ***In silico*** **analysis of AtWWP1 protein** (A) Phylogenetic tree representing the predicted relationship between AtWWP1 (AT2G41020) and WW domain-containing proteins in agronomic crops and Arabidopsis. The AtWWP1 clade is highlighted in red. (B) Phylogenetic tree of WW-domain-containing proteins from Arabidopsis. The AtWWP1 clade is highlighted in grey. The amino acid sequences containing WW domain were aligned using MUSCLE. Phylogenetic trees (A) and (B) were constructed using Bayesian inference performed with MrBayes v3.2.2 with the mixed amino acid substitution model (Jones). (C) Multiple alignment of AtWWP1 with Prp40 proteins from Arabidopsis. The WW domain regions are highlighted in grey. (D) Sequence alignment of AtWWP1 and human PQBP1. A C-terminus conserved motif is indicated in dark grey and a WW domain in light gray. Both alignments, (C) and (D), were carried out with CLUSTAL OMEGA. Identical amino acids are indicated with asterisks, highly conserved residues with (**:**) and weakly conserved (**.**)

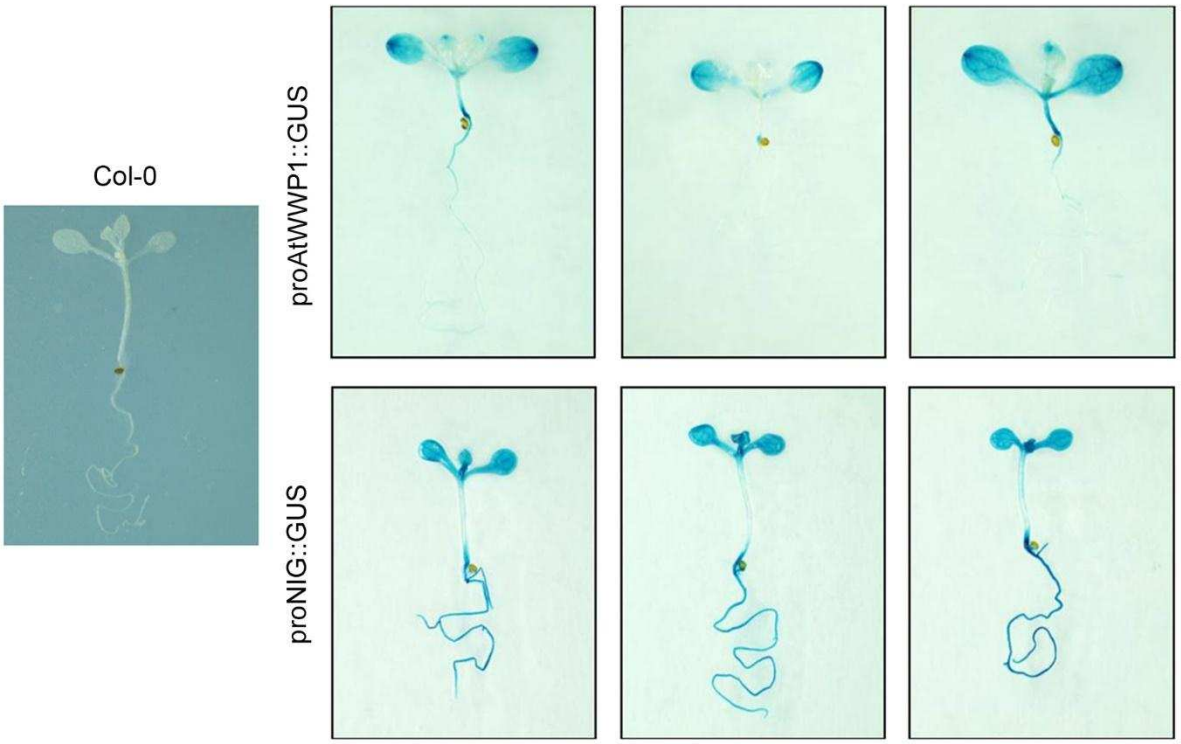

Figure S2. Tissue-specific expression of the AtWWP1 promoter.
GUS reporter gene expression was histochemically monitored in 2-week-old seedling leaves and roots from transgenic lines harboring a β-glucuronidase (GUS) reporter gene expressed from the AtWWP1 and NIG promoters. The figure shows histochemical analysis of GUS activity of three independent lines.

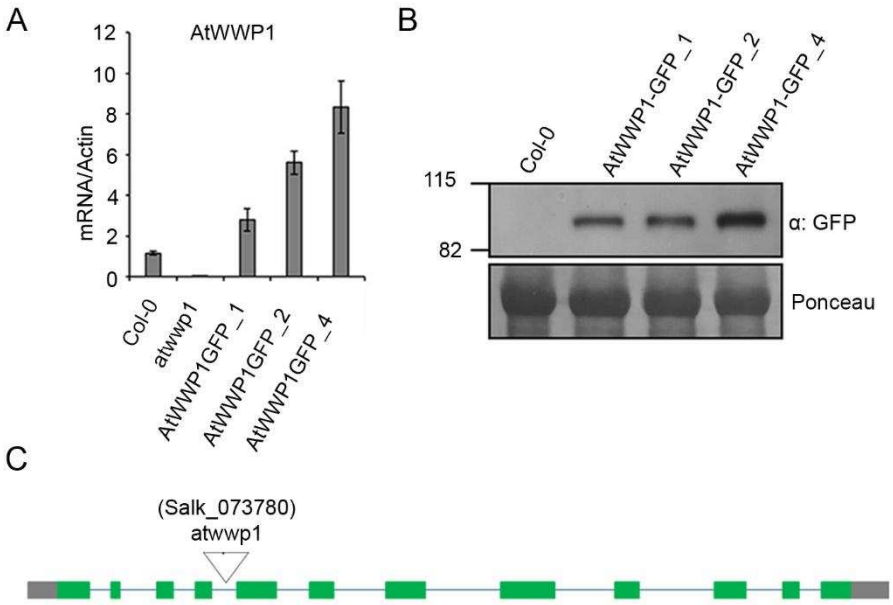

Figure S3. AtWWP1 transcript levels and protein accumulation in transgenic lines
(A) Accumulation of AtWWP1 transcript levels in wild type and transgenic lines, monitored by qRT-PCR and normalized to actin. Error bars, 95% confidence intervals (n=3) based on bootstrap resampling replicates of three independent experiments.
(B) Accumulation of AtWWP1-GFP protein levels in Col-0, AtWWP1 ovexpressing lines and atwwp1 plants. Protein extracts were immunoblotted with anti-GFP antibodies.
(C) Schematic representation of T-DNA insertion site in *atwwp1* (Salk_073780). Triangles show the positions of the T-DNA insertion in AtWWP1 sequence. The thick lines depict introns, green boxes mean exons. Grey boxes represent the 5′- and 3′-UTRs.

AtWWP1 – overexpressing line 4

atwwp1 knockout line

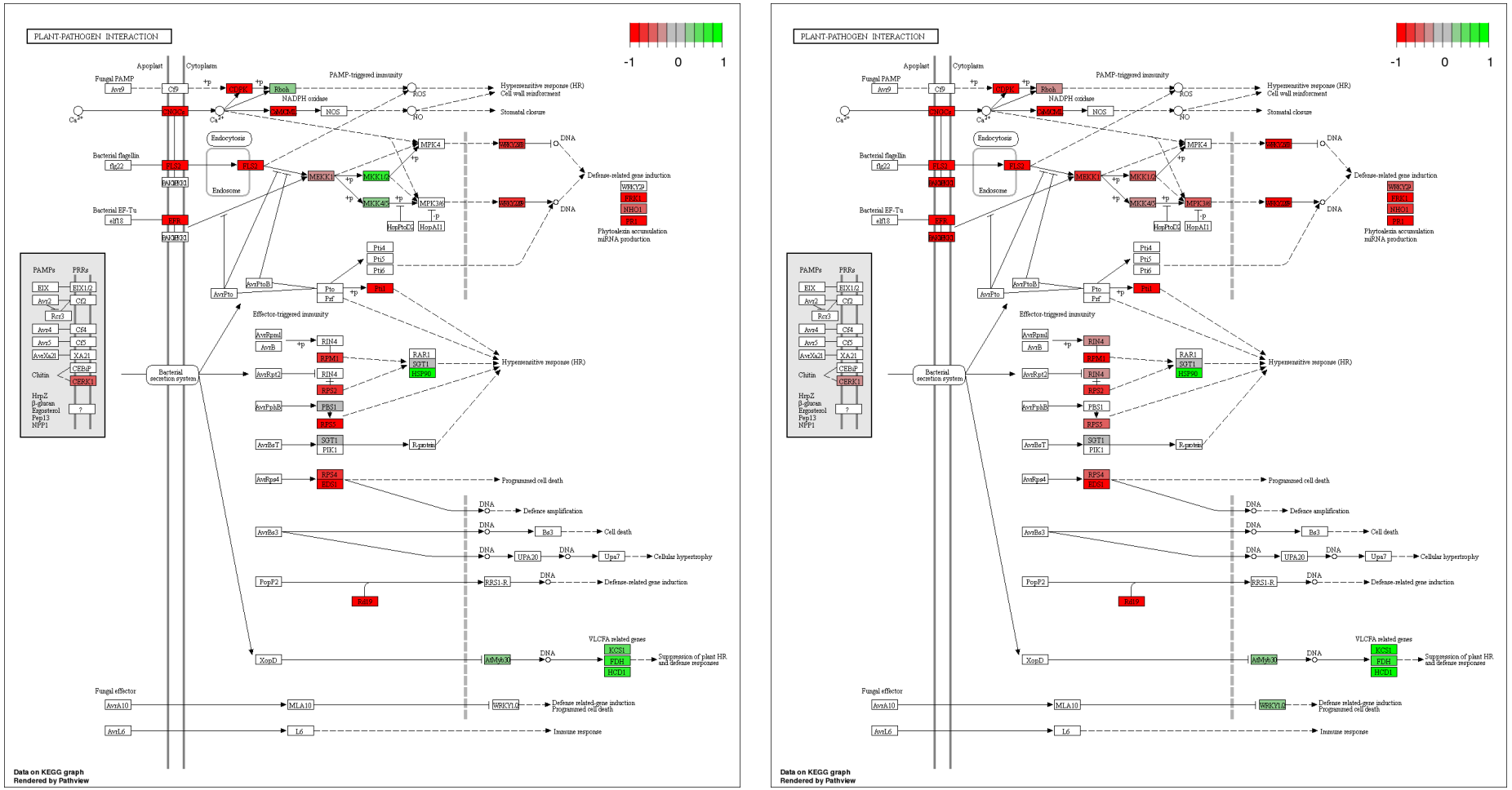

Figure S4. General downregulation of differentially expressed genes related to immune response in AtWWP1ox and atwwp1 mutant lines. Schematic representation of Plant-Pathogen Pathway view. Up- and down-regulated genes in Arabidopsis *AtWWP1*-overexpressing line-4 and *atwwp1* mutant are shown in green and red, respectively.

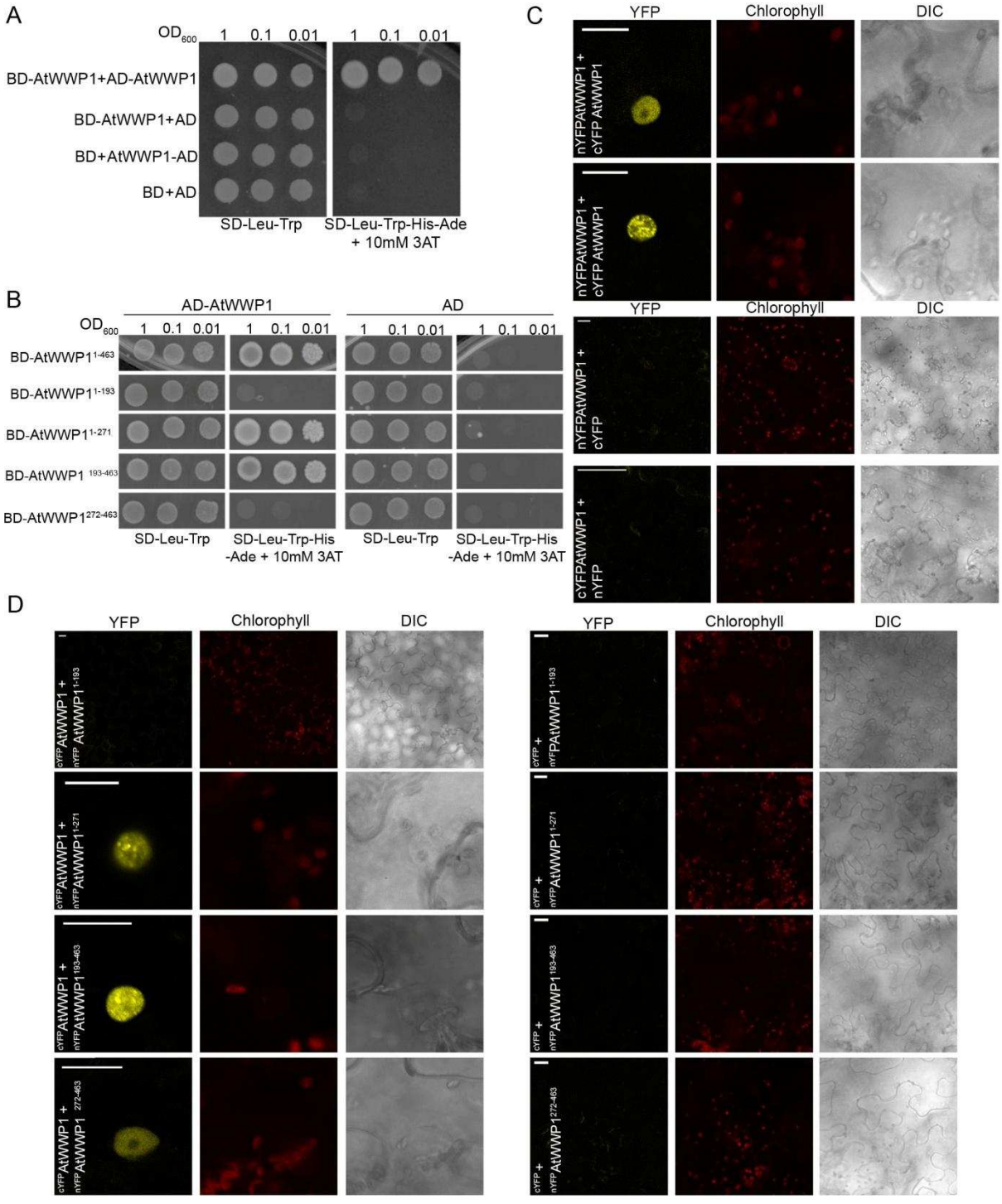
A
OD600
1 0.1 0.01 1 0.1 0.01
BD-AtWWP1+AD-AtWWP1
BD-AtWWP1+AD
BD+AtWWP1-AD
BD+AD
SD-Leu-Trp
SD-Leu-Trp-His-Ade + 10mM 3AT
B
AD-AtWWP1
AD
OD600
1 0.1 0.01 1 0.1 0.01 1 0.1 0.01 1 0.1 0.01
BD-AtWWP1 1-463
BD-AtWWP1 1-193
BD-AtWWP1 1-271
BD-AtWWP1 193-463
BD-AtWWP1 272-463
SD-Leu-Trp
SD-Leu-Trp-His -Ade + 10mM 3AT
SD-Leu-Trp
SD-Leu-Trp-His -Ade + 10mM 3AT
C
YFP
Chlorophyll
DIC
nYFPAtWWP1 + cYFP AtWWP1
nYFPAtWWP1 + cYFP AtWWP1
YFP
Chlorophyll
DIC
nYFPAtWWP1 + cYFP
cYFPAtWWP1 + nYFP
D
YFP
Chlorophyll
DIC
cYFP AtWWP1 + nYFP AtWWP1 1-193
cYFP AtWWP1 + nYFP AtWWP1 1-271
cYFP AtWWP1 + nYFP AtWWP1 193-463
cYFP AtWWP1 + nYFP AtWWP1 272-463
YFP
Chlorophyll
DIC
cYFP + nYFP AtWWP1 1-193
cYFP + nYFP AtWWP1 1-271
cYFP + nYFP AtWWP1 193-463
cYFP + nYFP AtWWP1 272-463

Figure S5. AtWWP1 forms dimmers through its WW domain.

(A) Yeast two-hybrid assay of AtWWP1 fused to GAL4 domains. AtWWP1-AD and AtWWP1-BD were expressed in yeast and assayed for interaction on selective medium supplemented with 3AT.

(B) Yeast two-hybrid analysis of association between AtWWP1 and its truncated forms. $AtWWP1^{1-193}$, $AtWWP1^{1-271}$, $AtWWP1^{193-463}$ and $AtWWP1^{272-463}$ fragments were fused to GAL4 BD domains and co-expressed in yeast with AD-AtWWP1 construction. The interactions between the tested proteins were analyzed on selective medium supplemented with 3AT.

(C) *In vivo* AtWWP1 self-interaction. *N. benthamiana* leaves were co-infiltrated with the constructions cYFP-AtWWP1, nYFP-AtWWP1 or empty vectors and examined using the confocal microscope 3 day after infiltration. Scale bars, 10 μm.

(D) Interaction analysis between AtWWP1 and AtWWP1-truncated forms. $AtWWP1^{1-193}$, $AtWWP1^{1-271}$, $AtWWP1^{193-463}$ and $AtWWP1^{272-463}$ truncated forms were fused to YFP N-terminal (nYFP) and co-expressed in *N. benthamiana* leaves with cYFP-AtWWP1 or empty vector. The images were acquired 3 days after infiltration using a confocal microscope. Scale bars, 10 μm.

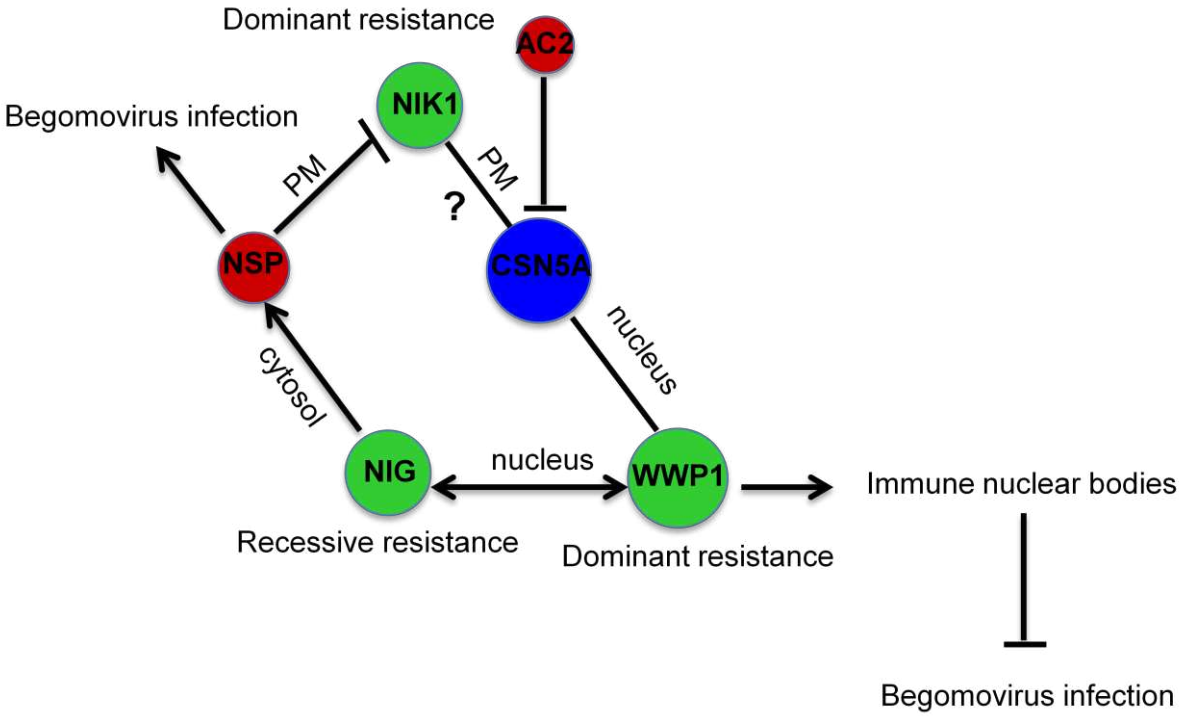

Figure S6. Begomovirus-Interacting Immune hub
Schematic representation of previously identified interactions involving begomovirus proteins (red) and host proteins (green) converging to CSN5A (blue), the central component of the immune hub. (Carvallho *et al*., 2008; Fontes *et al*.,2004; Lozano-Durán *et al*., 2011; Mukhtar *et al*., 2011).

Chapter III

THE IMMUNE ATWWP1- NUCLEAR BODIES REPRESENT HOTSPOTS FOR ACTIVE TRANSCRIPTION

THE IMMUNE ATWWP1- NUCLEAR BODIES REPRESENT HOTSPOTS FOR ACTIVE TRANSCRIPTION

Abstract

AtWWP1 is a WW domain-containing protein identified as a cellular partner of NIG, a cytosolic GTPase targeted by the viral movement protein NSP from begomoviruses. AtWWP1 forms nuclear bodies (NBs) via its WW domain and tethers NIG into this sub-nuclear structure, disturbing the NIG pro-viral function, thereby affecting negatively begomovirus infection. According to the current hypothesis, AtWWP1 organizes nuclear foci, which provide intrinsic immunity against begomovirus infection. Nevertheless, the underlying biochemical function of these AtWWP1-NBs has yet to be elucidated. In this investigation, we demonstrated that AtWWP1-NB co-localizes with CDKC2-NB, which has been shown to be involved in transcription and RNA processing. Like CDKC2, which modulates the phosphorylation status of RNA polymerase II C-terminus domain (CTD-RNAPII), we showed that AtWWP1 interacts with CTD-RNAPII within NBs. AtWWP1-NBs were disintegrated into diffuse pattern or converted to larger structures upon treatment with phosphorilation and transcriptional inhibitors, a feature that resembles the CDKC2-NB dynamic organization, which depends on the transcriptional status of the cells. As further evidence for a role in transcription, we also demonstrated that AtWWP1 displays DNA binding activity and AtWWP1-NBs associate with active chromatin regions. Accordingly, the manipulation of the AtWWP1 levels promoted an enrichment of differentially expressed transcriptional factors in transgenic lines. Collectively, our data

establish that the nuclear bodies formed by AtWWP1 are associated with active gene expression or co-transcriptional RNA processing.

Introduction

The cell nucleus is a complex and highly dynamic organelle that represents a site for a diverse array of cellular activities, including chromosome organization, DNA replication, RNA synthesis and processing, ribosome subunit biogenesis (Dundr and Misteli, 2010). Since the nucleus orchestrates such a range of biological processes, its spatial regulation is crucial for cell survival and homeostasis.

Nuclear bodies are dynamic nuclear domains, which contain an expanding list of enriched components with conserved functions across many species and provide a specialized environment for a variety of biological processes (Dundr, 2012; Sawyer and Dundr, 2016). These structures include nucleoli, Cajal bodies (CBs), histone locus bodies (HLBs), splicing factor compartments (speckles or interchromatin granule clusters), paraspeckles, promyelocytic leukemia (PML) bodies and protein degradation bodies (clastosome; Mao *et al.*, 2011). In plants, these subnuclear structures have been implicated in several hormone- and light-regulated signaling pathways, as well as with cicardian clock components. Nuclear bodies have also been reported to be associated with plant immunity (Shaw and Brow, 2004; Padmanabhan *et al*., 2013; Harris *et al*., 2016).

Recently, a nuclear body-forming protein, designated AtWWP1, has been described as a component of plant immunity against viruses (Chapter II). AtWWP1 harbors two WW domains and mediates subcellular redistribution of NIG, a host protein targeted by the viral movement protein NSP. Consistent with an antiviral function, *AtWWP1* overexpression confers tolerance to *Begomovirus*, a widespread DNA virus, and the AtWWP1 nuclear bodies (AtWWP1-NB) seem to be dynamically changed upon virus infection. However, the cellular function of AtWWP1 remains to be determined.

Due its potential relevance in antiviral immunity, we attempted to elucidate the biological role of AtWWP1, as well as the biological significance of AtWWP1-NBs. We demonstrated that AtWWP1 co-localizes with CDKC2-NBs, interacts with the C-terminus domain of RNA polymerase II (CTD-RNAPII), as well as with single-stranded (ssDNA) and double-stranded DNA (dsDNA). In addition, AtWWP1 is associated with active chromatin in the nucleus and its nuclear bodies change dynamically upon alterations in the (de)phosphorylation *status* and the transcription level of the cell. Our data suggest that AtWWP1-NB may play a transcriptional regulatory role and hence might represent an active immune signaling site against virus.

Results

AtWWP1 co-localizes with the Cyclin-dependent kinase CDKC2, a transcription/splicing-associated nuclear body marker

Many cellular processes, including DNA replication, splicing and protein degradation, are known to be regulated trough compartmentalization in the nucleus (Shaw and Brown, 2004). AtWWP1 has been shown to form nuclear bodies (NBs) varying in number and size, as well as displaying a diffused nucleoplasmic pattern (Chapter II). To understand the molecular composition of AtWWP1-NBs, we performed co-localization studies with well-established markers of known subnuclear compartments in Arabidopsis, including coilin (Cajal body component), CypRS64 and SR34 (splicing), AtGRP7 (circadian rhythm), fibrillarin (nucleolar pre-rRNA splicing) and CDKC2 (splicing and transcription; Boudonck *et al*., 1999; Kitios *et al*, 2008; Lorković *et al*., 2008). We transiently co-expressed AtWWP1-GFP with the protein markers fused to mCherry fluorescent tag in *Nicotiana benthamiana* leaves and analyzed the subnuclear localization of both proteins by confocal microscopy (Fig. 1). No detectable co-localization overlap was observed between AtWWP1 and Fibrillarin, Coilin, SR34, AtGRP7 or CypRS64 marker proteins (Fig.1). These data suggest that AtWWP1-NBs are distinct from these previously characterized subnuclear compartments; thereby, AtWWP1 may have little functional relationship with the cellular processes associated with these NB markers. In contrast, a partial overlapping in nuclear distribution was observed when AtWWP1 was co-expressed with CDKC2.

Cyclin-dependent kinase Cs (CDKCs) are known to be involved in transcriptional events by modulating the C-terminus domain (CTD) of RNA polymerase II (RNAP II) activity trough phosphorylation. Arabidopsis CDKC1 and CDKC2, homologs of mammalian CDK9 and CRK7, phosphorylate the CTD *in vitro* (Cui *et al*., 2007; Hajheidari *et al*., 2013). Additionally, CDKC2 has been

show to co-localize with spliceosomal components in nuclear speckles (Kitsios *et al*., 2008), which indicates that CDKC2 is probably involved in splicing events. The co-localization between AtWWP1 and CDKC2 suggests that these proteins may concentrate in the same functionally related microdomain. Thus, AtWWP1 may have a role in transcription and RNA processing.

AtWWP1 is an RNA polymerase II carboxyl-terminus domain (CTD)-associated protein

In a previous phylogenetic analysis, AtWWP1 was grouped with a set of Arabidopsis CTD-associated proteins (Chapter II, Kang *et al*., 2009). Similarly, the AtWWP1 human homologous PQBP1 is a CTD partner (Chapter II; Okazawa *et al*., 2002). To uncover the cellular function of AtWWP1, we first examined its putative association with the CTD-RNAPII using yeast two-hybrid approach (Fig. 2A). Full-length *AtWWP1* coding region and the CTD domain of RNAP II (NRPB1 encodes the largest subunit of RNAP II) were fused to the activation domain (AD) and the binding domain (BD) of GAL4 and expressed in a yeast strain harboring a *HIS3* reporter gene. Co-expression of AD-AtWWP1 and BD-CTD-RNAPII in yeast activated the *HIS3* reporter gene and promoted prototrophy in medium lacking histidine, while the yeast transformed with respective controls did not display growth (Fig. 2A). We further investigated the association between AtWWP1 and CTD-RNAPII *in vivo* using BiFC assay (Fig. 2B). AtWWP1 and CTD-RNAPII were fused to non-fluorescent fragments (N-terminus and C-terminus) from YFP and expressed in *N. benthamiana* leaves via *Agrobacterium tumefaciens*. YFP reconstituted fluorescence was observed in cells co-expressing WWP1- cYFP and CTD-RNAPII-nYFP constructs, but not

in cells co-expressing WWP1-cYFP and CTD-RNAPII-nYFP with the respective empty vectors. The YFP fluorescence was observed in the nucleus of transfected cells as a diffused or punctuate pattern (Fig. 2B). Collectively, these data indicate that AtWWP1 is a CTD RNAPII-interacting protein.

Inhibition of transcription and (de)phosphorylation compromise AtWWP1 nuclear body distribution

AtWWP1 displays different subnuclear localization patterns, which suggest that its localization may change under different conditions. To understand the dynamics of AtWWP1 nuclear bodies, we examined the subnuclear localization of AtWWP1 after treatments with drugs that inhibit transcription (5,6-Dichloro-1-b-D-ribofuranosylbenzimidazole, DRB; Actinomicin-D), protein kinases (staurosporine) and phosphatases (okadaic acid) in Arabidopsis *AtWWP1*-overexpressing transgenic lines (Fig. 3A). Treatments with Actinomicin-D, staurosprine and okadaic acid compromised the AtWWP1 nuclear body-forming activity, resulting in a disorganized and dispersed pattern in the nucleus. Specifically, the inhibition of RNA polymerase II by DRB treatment resulted in the redistribution of AtWWP1 to enlarged bodies, resembling the CDKC2 redistribution pattern into larger nuclear inclusions by inhibitors of transcription (Kitsios *et al*., 2008). No effect was observed when *AtWWP1*-overexpressing lines were incubated with the control solvent DMSO. The redistribution of AtWWP1-NBs by inhibitors of transcription and (de)phoshorylation suggests that these subnuclear structures are functional and regulated by the phosphorylation/dephosphorylation level and the transcriptional status of the cell.

AtWWP1 is associated with active chromatin

The association between AtWWP1 and CTD-RNAPII, along with the AtWWP1- and CDKC2-NBs co-localization, and interference of AtWWP1 distribution by the level of RNAPII transcription *in vivo*, raised up the hypothesis that AtWWP1 would take part in transcription and RNA processing. In higher eukaryotes, the transcriptional machinery has been demonstrated to be compartmentalized into subnuclear structures (Papantonis and Cook, 2013; Allen and Taatjes, 2015); thereby, the AtWWP-NBs would be associated with transcriptionally active foci and excluded from the heterochromatin region. To address this issue, we investigated the association of AtWWP1 with heterochromatin regions in the nucleus using the DAPI marker. In plants, DAPI stains chromocenters and highly compacted chromatin regions with limited transcriptional activity (van Zanten *et al*., 2012; Kaiserli *et al*., 2015). Subnuclear co-localization studies demonstrated that AtWWP1-NBs were not associated with heterochromatin regions because there was no overlap of AtWWP1-GFP with DAPI microdomains (Fig. 3B). Collectively, our data indicate that AtWWP1-NBs are functionally associated with active chromatin and may be involved in co-transcriptional RNA processing and gene expression. Consistent with this hypothesis, overexpression or inactivation of *AtWWP1* function promoted an enrichment of differentially expressed transcriptional factors (TFs; Table S1 and S2; http://inctipp.bioagro.ufv.br/AtWWP1_genome/). In the *atwwp1* lines, there was a remarkably enrichment of transcriptional factor activity, which was overrepresented in the down-regulated changes (125 down-regulated x 100 up-

regulated genes). In contrast, in the AtWWP1-4 line, all differentially expressed TFs (129) were up-regulated. This major down-regulation of TFs in *atwwp1* lines along with the up-regulation of TFs in the *AtWWP1*-overexpressing line further suggests that AtWWP1-NBs may positively regulate transcription.

Important cellular processes, such as DNA replication and repair or RNA transcription and processing are organized in nuclear subdomains (Lamond and Spector, 2003). To provide insights into the biological function of AtWWP1, we investigate the nucleic acid binding properties of AtWWP1 (Fig. 4). To assay for DNA binding activity, the recombinant haemagglutinin(HA)-fused proteins were transcribed and translated *in vitro* (input) and incubated with ssDNA or dsDNA linked to cellulose beads. RNA binding activity was monitored by incubating the *in vitro* translated GST-fused proteins with A. *thaliana* biotinylated RNA conjugated with streptavidin beads. We also included the histone 3 (H3, AT4G40030) and PIF4 as positive controls for DNA binding assays and AtGRP7 (Heintzen *et al*., 1997) as a positive control for RNA binding activity. As negative controls, we assayed HA and GST tags alone. AtWWP1-HA fusion protein was found to bind either to single-stranded (ss) and double-stranded (ds) DNA (Fig. 4A, Fig. S1), but not to RNA (Fig. 4B). NIG, an AtWWP1 partner, did not display DNA binding activity *in vitro* (Fig. S1). AtMBD2, a methyl-CpG-binding protein, possesses DNA binding properties (Fig. S1), but did not interact with RNA (Fig. 4B). These results further support the argument that that AtWWP1-NBs are functionally associated with active chromatin and may be involved in transcription.

AtWWP1 is connected to a transcription factor hub related to auxin signaling

WW domain-containing proteins are known to mediate protein-protein interactions via their WW domain. Many of WW domain-binding partners are components of multiprotein complexes involved in molecular processes, such as transcription, RNA processing, and cytoskeletal regulation (Ingham *et al*., 2005). Thus, WW domains can yield a functional network of protein interactions involved in a wide range of biological processes. According to the Arabidopsis interactome *network*, AtWWP1 interacts with AtMBD2, a methyl CpG binding domain-containing protein. AtMBD2, in turn, is directly connected to an auxin hub of transcription factors (Fig. S2). The interaction between AtWWP1 and AtMBD2 was examined by yeast two-hybrid and confirmed *in vivo* by CoIP and BiFC (Fig. 5). Co-expression of BD-AtMBD2 and AD-AtWWP1 activated both *HIS3* and *β-Gal* reporter genes (Fig. 5A and 5B), which was in marked contrast with the negative controls (empty vectors). We also noticed that AtWWP1 displays transcriptional activation activity in yeast, because BD-AtWWP1 alone is able to transactivate the reporter genes (Fig. 5A and 5B), although to a lesser extent than the transcriptional activator NIG (compare yeast growth in selective medium and *β*-galactosidase activity of BD-NIG transformed cells with those of BD-AtWWP1 + pAD co-transformed cells). The *in vivo* interaction between AtWWP1 and AtMBD2 was confirmed by Co-IP (Fig. 5C). BiFC assay demonstrated that AtWWP1 and AtMBD2 interacted in nuclear bodies (Fig. 5D). Consistently, AtWWP1 also relocates AtMBD2 to nuclear bodies when co-expressed in *N. benthamiana* leaves (Fig. 5E). Differently from AtWWP1-NIG and AtWWP1-CTD-RNAPII interactions, which involve the WW domains, the association between AtMBD2 and AtWWP1 is mediated by the C-terminus

domain on AtWWP1 (Fig. S3), indicating that the functionality of AtWWP1 is not restricted to the WW domain modules. Recently, we have shown that the C-terminus domain of AtWWP1 is capable of promoting weak intramolecular interactions and, hence, may harbor another protein-interacting module (Chapter II). AtWWP1 may act as a scaffold protein, recruiting factors into transcriptionally active nuclear bodies.

Discussion

Previous studies have demonstrated that AtWWP1 displays a role in plant viral immunity, which may be associated with its nuclear body-forming capacity (Chapter II). In fact, AtWWP1 nuclear bodies sequester host factors targeted by plant virus. A well-characterized example is NIG, an NSP-interacting partner, which is forced by AtWWP1 to change its subcellular localization from the cytoplasm to the nucleus, where it concentrates in AtWWP1 nuclear bodies (Chapter II). In the current investigation, we identified another host factor CDKC2, which shares the same nuclear microdomain with AtWWP1-NBs (Fig. 1). CDKC2, which phosphorylates the CTD-RNAPII domain, plays an important role in *Cauliflower mosaic virus* (CaMV) infection. CaMV is a dsDNA virus that depends on host RNAPII for transcription; thereby, the CDKC kinase complexes may act as important host targets of CaMV for transcriptional activation of viral genes. Accordingly, it has been demonstrated that CaMV 35S promoter activity is reduced in *cdkc2* knockouts. Arabidopsis *cdkc2* mutants are more resistant to CaMV infection, as compared to wild type (Cui *et al*., 2007). CDKC2 has also

been reported to co-localize with spliceosomal components in nuclear speckles, which are dependent on the transcriptional status of the cell (Kitsios *et al*., 2008). The co-localization between AtWWP1 and CDKC2 indicates that AtWWP1 may be functionally associated with transcription and RNA processing. Consistent with this argument, our data demonstrated that AtWWP1 is a CTD-RNAP II-interacting protein (Fig. 2). As AtWWP1 displays a role in antiviral immunity (Chapter II), its direct and indirect association with CTD-RNAPII and CDKC2, respectively, may indicate that AtWWP1-NBs constitute an immune foci that modulate plant response against pathogens.

Recent studies have associated CTD phosphorylation dynamics with regulation of plant immunity (Li *et al*., 2014; Li *et al*., 2016). The flg22-induced MAPK cascade has been demonstrated to induce phosphorylation and activation of CDKCs (CDKC1 and CDKC2), which, in turn, phosphorylate the tail of CTD-RNAPII. CDKCs may positively regulate plant innate iImmunity, because *cdkc1* and *cdkc2* mutant plants are more susceptible to bacterial infection. Therefore, the CTD phosphorylation status is thought to recruit gene-specific transcription factors (TFs) in a regulatory manner and hence orchestrates the transcription of immune genes in PTI signaling (Li *et al*., 2014).

Consistently with the correlation between AtWWP1-NB and transcription sites, we observed a dynamic alteration in the AtWWP1-NB organization upon inhibition of phosphatases, kinases and transcription in Arabidopsis root cells (Fig. 3A). Treatment with transcription inhibitors caused the redistribution of AtWWP1 into enlarged bodies, resembling the pattern of CDKC2 redistribution upon transcription inhibition (Kitsios *et al*., 2008). Similarly to the AtWWP1-NB organization, treatment with okadaic acid and staurosporine (Ser/Thr

phosphatase and kinase inhibitors, respectively) resulted in both a diffuse pattern of the splicing factor AtRSp31 and its concentration into larger speckles around the nucleolus (Docquier *et al*., 2004). Overall, several lines of evidence indicate that phosphorylation and transcription regulate the distribution and mobility of plant splicing factors in the same manner as observed here for the AtWWP1 nuclear distribution (All and Reddy, 2006; Tillemans *et al*., 2005; 2006).

In addition to splicing components, transcription-regulated factors were also been reported to occupy nuclear subdomains. In Arabidopsis, a common feature of numerous light and clock signaling proteins is their localization within nuclear bodies (Herrero and Davis, 2012). Many cicardian-clock components, which act in the nucleus as transcriptional regulators, co-localize in nuclear foci. A key component of the cicardian clock, GIGANTEA, co-localizes in nuclear bodies with other flowering-related proteins and contributes to the regulation of the photoperiodic flowering in Arabidopsis (Kim *et al*., 2013). Arabidopsis light components localize in nuclear bodies (photobodies), which are dynamically modified under different light stimuli (Kircher *et al.*, 2002; Chen *et al*., 2003; Chen and Chory, 2011). The receptors phytochromes A and B (phyA and phyB) are dynamically localized in nuclear bodies where they regulate the abundance and activity of different transcription factors (Leivar *et al*., 2011; Kaiserli *et al.,* 2015). Recent studies have demonstrated that nuclear photobodies constitute active sites of transcription (Kaiserli *et al*., 2015). TZP (Tandem Zinc-finger-Plus3), a positive regulator of the photoperiodic flowering, is recruited by phyB into a transcriptionally active nuclear photobodies. Treatment of TZP-NB with the transcriptional blockers Actinomycin D and DRB resulted in the formation of

one larger TZP nuclear aggregate (Kaiserli, *et al*., 2015). Therefore, the reorganization of typical nuclear bodies into larger structures mediated by transcription inhibition seems to be a feature of transcriptionally active nuclear foci and further substantiates the notion that AtWWP1-NBs represent transcriptionally active microdomains in the nucleus.

The assignment of AtWWP1-NBs as transcriptional nuclear microdomains was further complemented with experiments demonstrating that AtWWP1-NBs associate with active chromatin (Fig. 3B) and AtWWP1 displays DNA-binding activity (Fig. 4A). Additionally, overexpression or inactivation of *AtWWP1* function promoted an enrichment of differentially expressed transcriptional factors, which were predominantly down-regulated in the knockout line and up-regulated in the overexpressing AtWWP1-4 line (Table S1 and S2). We also demonstrated that AtWWP1 is connected to a transcription factor hub related to auxin signaling via association with AtMBD2 and to an immune hub via association with NIG (Fig. 5 and Fig. S2). Collectively, our data strongly indicate that AtWWP1 forms immune nuclear bodies that demarcate a transcriptionally active site in the nucleus of plant cells.

Materials and Methods

Plasmid constructions

All recombinant plasmids were generated by the GATEWAY cloning system (Invitrogen, http://www.invitrogen.com/). The protein AtMBD2, encoded

by the AT5G35330 locus, was identified as a pontential interacting partner of AtWWP1 (http://signal.salk.edu/interactome/AI1.html). AtMBD2 coding regions either with (ST) or without (NS) a translational stop codon were amplified from cDNA of Arabidopsis ecotype Columbia and inserted into the entry vectors pDONR201 and pDONR207 using the primers AtMBD2-Fwd AAAAAGCAGGCTTCACAATGAGTATGTCGCAGTC, AtMBD2-ST-Rvs AGAAAGCTGGGTCTTATCTATCAGCAAGTTCGTC, AtMBD2-NS-Rvs AGAAAGCTGGGTCTCTATCAGCAAGTTCGTC. The resulting clones were designated AtMBD2ST-pDONR201 (pUFV1987), AtMBD2NS-pDONR201 (pUFV1988) and AtMBD2NS-pDONR207 (pUFV2054). The C terminus domain of RNA polymerase II was isolated from *NRPB1*, which encodes the largest subunit of RNAP II, using the primers CTDRNApolIII–Fwd AAAAAGCAGGCTTCACAATGTCCCCAATGTCAGA, CTDRNApolIII–stRvs AGAAAGCTGGGTCTCAAGGGTTGCCTTTATCATC and CTDRNApolIII–nsRvs AGAAAGCTGGGTCAGGGTTGCCTTTATCATC. The resulting clones were designated CTD-RNAPIIST-pDONR201 (pUFV2271) and CTD-RNAPIINS-pDONR207 (pUFV2314).

For yeast two-hybrid assay, AtMBD2 and CTDRNAPII coding regions were transferred from the respective previously described entry clones to the yeast expression vectors pDEST32 and pDEST22, generating the clones pBD-AtMBD2 (pUFV2172), pAD-AtMBD2 (pUFV2173) and pBD-CTDRNAPII (pUFV2274) as GAL4 binding domain (BD) or activation domain (AD) fusions.

For subcellular localization, co-immunoprecipitation and BIFC assays, AtMBD2 and CTDRNAPII coding regions were transferred to different destination vectors for expression in plants (pK7FWG2, SPYNE-GW and

SPYCE-GW). AtMBD2 coding region was fused to the fluorescent tags GFP and mCherry and expressed in plants driven by 35S CaMV promoter. The resulting recombinant constructions were designated 35S::AtMBD2-GFP (pUFV1997), 2x35S::AtMBD2-mCherry (pUFV2180). For BiFC, the insert of AtMBD2NS-pDONR207 was transferred to SPYNE-GW and SPYCE-GW resulting in AtMBD2-nYFP (pUFV pUFV2055) and AtMBD2-cYFP (pUFV2056). The CTDRNAPII domain was cloned into SPYNE and SPYCE vectors, generating the clones CTDRNAPII-nYFP (pUFV2319) and CTDRNAPII-cYFP (pUFV2318). All AtWWP1, AtWWP1 truncated forms, and NIG constructions used for subcellular co-localization, yeast two-hybrid, co-immunoprecipitation and BiFC assays are described in Chapter II.

For *in vitro* protein expression, the AtWWP1, NIG and AtMBD2 coding regions were transferred from the pDONR entry vector to pTNT-GSTGW and pIXHAGW vectors, which allow the expression of recombinant proteins fused to GST (Glutationa-S-transferase) and HA (Haemaglutinin) tags, respectively. The resulting clones were designated as pTNT-GST-AtWWP1 (pUFV2175), pTNT-GST-AtMBD2 (pUFV2177), pIXHA-NIG (pUFV2178), pIXHA-AtWWP1 (pUFV2174) and pIXHA- AtMBD2 (pUFV2176).

Yeast two-hybrid assay

Saccharomyces cerevisiae AH109 strain was co-transformed with the yeast expression vectors pDEST22 (Gal4 AD) and pDEST32 (Gal4 DB) harboring the coding region of tested proteins by the lithium acetate/polyethylene glycol method. Transformed yeast cells bearing both plasmids were selected on synthetic dropout medium (SD) lacking Trp, Leu for

4 days at 28 °C. Specific interactions were tested by growing transformed cells on SD medium lacking Trp, Leu, His or Trp, Leu, His, supplemented with 3-amino-1,2,4-triazole (1 to 10 mM 3AT) to monitor the strength of the interaction. Quantitative measurements of β-galactosidase activity in yeast extracts were monitored using o-nitrophenyl β-D-galactopyranoside (ONPG) assay, as described previously (Uhrig *et al*., 1999).

Co-immunoprecipitation assay

The *in vivo* interactions between AtWWP1 and AtMBD2 were assayed by co-immunoprecipitation using the μMACS Epitope Tag Protein Isolation Kit (MACS/Miltenyi Biotec), according to the manufacturer's instructions. Transient *Agrobacterium*-mediated expression in *N. benthamiana* leaves was performed, as previously described by Carvalho *et al*. 2008. Total protein extracts were obtained from infiltrated leaves expressing the tested recombinant proteins and incubated for 2 h with anti-GFP magnetic beads (MACS/Miltenyi Biotec) at 4 ºC under gentle rotation. After the incubation step, the extracts were applied into a MACS column placed in the indicated separator and washed five times for removal of unbound material. The elution step was performed using 50 μL of elution buffer pre-warmed to 95 ºC. Eluted fractions were resolved on a 10% (w/v) SDS-PAGE gel, immunoblotted with anti-HA (Miltenyi Biotec, catalogue number 130-091-972) or anti-GFP (Miltenyi Biotec, catalogue number 130-091-833) monoclonal antibodies. The reacting antibodies were detected using Signal West Pico Chemiluminescent Substrate (Thermo Scientific), according to the manufacturer's instructions.

Bimolecular fluorescence complementation (BiFC)

Different combinations of the *A. tumefaciens* strain GV3101 harboring the indicated nYFP/cYFP-fused protein constructs were co-infiltrated into the abaxial surface of *N. benthamiana* leaves at an OD_{600nm} ratio of 1:1 for infiltration. Fluorescence was analyzed in epidermal cells 3 days after infiltration using a Zeiss inverted LSM510 META laser scanning microscope equipped with an argon laser and a helium laser as excitation source. YFP was excited at 514nm using an argon laser, and YFP emission was detected using a 560-615nm filter.

Subcellular localization

Subcellular and subnuclear localizations of fluorescence fusion proteins were analyzed by transient expression in *N. benthamiana* leaves. *A. tumefaciens* GV3101 was transformed with DNA constructions of the tested protein fused to GFP, YFP or mCherry fluorescent tags. Fluorescence was visualized in epidermal cell layers of the leaves after 3 day of infiltration using a confocal microscopy. For co-localization studies of AtWWP1 with subnuclear markers, the Fibrilarin (AT4G25630), Coilin (AT1G63780), AtGRB7 (AT2G21660), CypRS64 (AT3G63400), CDKC2 (AT5G64960) and SR34 (AT1G02840) coding regions fused to mCherry were donated by Dr Joanne Chory from Plant Molecular Biology Lab/Salk Institute.

Stable transgenic lines expressing the recombinant fluorescent proteins were also assayed for subcellular localization. The transgenic lines were obtained by *Agrobacterium*-mediated transformation using the floral dip method (Zhang *et al*., 2006). Root fragments from different Arabidopsis transgenic

seedlings were imaged by confocal microscopy. For imaging GFP, the 458nm excitation line and the 500 to 530 nm band pass filter were used. Excitation of YFP was at 514nm and YFP emission was detected by using a 560–615 nm filter. Excitation of mCherry was at 540nm and emission of 608-680 nm.

In vitro Nucleic Acid binding assay

Full-length tested proteins AtWWP1, AtMBD2, NIG and control proteins, including histone H3 (AT4G40030), AtGRB7 (AT2G21660) and PIF4, were fused to HA or GST tag and expressed *in vitro* using the TnT *in vitro* transcription/translation system (Promega). The DNA binding assay protocol was performed as described by Kaiserli *et al* (2015). Equal amounts of protein were incubated with either single-stranded or double-stranded deoxyribonucleic acid lyophilized powder attached to cellulose beads from calf thymus DNA (1 mg/ml). After incubation at 4 °C for 30 min, the beads were washed five times in RHPA buffer (10 mM Tris, pH 7.4, 2.5 mM MgCl2, 0.5% Triton X-100) and then boiled in SDS loading buffer. The proteins were separated by SDS-PAGE and detected by western blot using an anti-HA antibody. The RNA binding assay was modified from Vert and Chory (2006). Total RNA from *A. thaliana* was extracted and biotinylated using BrightStar Psolaren-Biotin kit, following the manufacture's instructions. For RNA–protein pulldown, biotinylated RNA was first incubated with streptavidin-bound beads (Dynabeads; invitrogen) in IP buffer (50 mM Tris-HCl pH 7.5, 100mM NaCl, 0.2% Nonidet P40) for 2 h at 4 °C; then, washed five times with IP buffer. Equal amounts of protein were then added to RNA-bound beads and incubated under rotation at 4 °C for 30 min. Subsequently, beads were washed five times with IP buffer, boiled in SDS

loading buffer and subjected to SDS-PAGE. The proteins were detected by western blot using an anti-HA or anti-GST antibody.

Inhibitor treatments

Arabidopsis 7-day-old transgenic seedlings overexpressing AtWWP1-GFP were treated with DRB (100 µg/ml), ActinomycinD (5 µg/ml), Staurosporine (10 µM) and okadaic acid (1 mM) as previously described (Tllemans *et al.*, 2006; Korelova *et al*., 2009). Control plants were treated with DMSO. After three hours of treatment, roots were imaged by confocal microscopy.

References

Ali, G.S., Reddy, A.S. (2006) ATP, phosphorylation and transcription regulate the mobility of plant splicing factors. *J. Cell Sci*. 119, 3527–3538.

Allen, B.L., Taatjes, D.J. (2015) The Mediator complex: a central integrator of transcription. *Nat. Rev. Mol. Cell Biol*. 16, 155-166.

Boudonck, K., Dolan, L., Shaw, P.J. (1999) The movement of coiled bodies visualized in living plant cells by the green fluorescent protein. *Mol. Biol. Cell* 10, 2297–2307.

Carvalho, C.M., Santos, A.A., Pires, S.R., Rocha, C.S., Saraiva, D.I., Machado, J.P., Mattos, E.C., Fietto, L.G., Fontes, E.P.B. (2008) Regulated nuclear trafficking of rpL10A mediated by NIK1 represents a defense strategy of plant cells against virus. *PLoS Pathog*. 4, e1000247.

Chen, M., Chory, J. (2011) Phytochrome signaling mechanisms and the control of plant development. *Trends Cell Biol*. 21, 664–671.

Chen, M., Schwab, R., Chory, J. (2003) Characterization of the requirements for localization of phytochrome B to nuclear bodies. *Proc. Natl. Acad. Sci. USA*, 100, 14493–14498.

Cui, X., Fan, B., Scholz, J., Chen, Z. (2007) Roles of *Arabidopsis* cyclin-dependent kinase C complexes in cauliflower mosaic virus infection, plant growth, and development. *Plant Cell*, 19, 1388–1402.

Docquier, S., Tillemans, V., Deltour, R., Motte P. (2004) Nuclear bodies and compartmentalization of pre-mRNA splicing factors in higher plants. *Chromosoma*, 112, 255–266.

Dundr, M. (2012) Nuclear bodies: multifunctional companions of the genome. *Curr. Opin. Cell Biol*. 24, 415–422.

Dundr, M., Misteli, T. (2010) Biogenesis of nuclear bodies. *Cold Spring Harb Perspect. Biol*. 2, a000711.

Endo, M. (2016) Tissue-specific circadian clocks in plants. *Curr. Opin. Plant Biol*. 29, 44–49.

Cui, X., Fan, B., Scholz, J., Chen, Z. (2007) Roles of *Arabidopsis* cyclin-dependent kinase C complexes in cauliflower mosaic virus infection, plant growth, and development. *Plant Cell*. 19,1388–1402.

Hajheidari M., Koncz, C., Eick, D. (2013) Emerging roles for RNA polymerase II CTD in *Arabidopsis*. *Trends Plant Sci*. 18, 633-643.

Harris, C.J., Husmann, D., Liu, W., Kasmi, F.E., Wang, H., Papikian, A., Pastor, W.A., Moissiard, G., Vashisht, A.A., Dang, J.L., Wohlschlegel, J.A., Jacobsen,

S. E. (2016) *Arabidopsis* AtMORC4 and AtMORC7 Form Nuclear Bodies and Repress a Large Number of Protein-Coding Genes. *PLoS Genet. 12*, e1005998.

Heintzen, C., Nater, M., Apel, K., Staiger, D. (1997) AtGRP7, a nuclear RNA-binding protein as a component of a circadian regulated negative feedback loop in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA*. 94, 8515-8520.

Herrero, E., Davis, S.J. (2012) Time for a nuclear meeting: Protein trafficking and chromatin dynamics intersect in the plant circadian system. *Mol Plant*, 5, 3, 554-565.

Ingham, R.J., Colwill, K., Howard, C., Dettwiler, S., Lim, C.S, Yu, J., Hersi, K., Raaijmakers, J., Gish, G., Mbamalu, G., Taylor, L., Yeung, B., Vassilovski, G., Amin, M., Chen, F., Matskova, L., Winberg, G., Ernberg, I., Linding, R., O'donnell, P., Starostine, A., Keller, W., Metalnikov, P., Stark, C., Pawson, T. (2005) WW domains provide a platform for the assembly of multiprotein networks. *Mol. Cell Biol*. 25, 7092–70106.

Kaiserli, E., Páldi, K., O'Donnell, L., Batalov, O., Pedmale, U.V., Nusinow, D.A., Kay, S.A., Choryet, J. (2015) Integration of light and photoperiodic signaling in transcriptional nuclear foci. *Dev. Cell*. 35, 311–321.

Kang, C.H., Feng, Y., Vikram, M., Jeong, I.S., Lee, J.R., Bahk, J.D., Yun, D.J., Lee, S.Y., Koiwa H. (2009) *Arabidopsis thaliana* PRP40s are RNA polymerase II C-terminal domain-associating proteins. *Arch. Biochem. Biophys*. 484, 30-38.

Kim, Y., Lim, J., Yeom, M., Kim, H., Kim, J., Wang, L., Kim, W.Y., Somers, D.E., Nam, H.G. (2013) ELF4 regulates GIGANTEA chromatin access through subnuclear sequestration. *Cell Rep*. 3, 671–677.

Kircher, S., Gil, P., Kozma-Bognár, L., Fejes, E., Speth, V., Husselstein-Muller, T., Bauer, D., Adám, E., Schäfer, E., Nagy, F. (2002) Nucleocytoplasmic partitioning of the plant photoreceptor phytochrome A, B, C, D, and E is regulated differentially by light and exhibit a diurnal rhythm. *Plant Cell*. 14, 1541–1555.

Kitsios, G., Alexiou, K.G., Bush, M., Shaw, P., Doonan, J.H. (2008) A cyclindependent protein kinase, CDKC2, colocalizes with and modulates the distribution of spliceosomal components in *Arabidopsis*. *Plant J*. 54, 220–235.

Koroleva, O.A., Brown, J.W., Shaw, P.J. (2009) Localization of eIF4A-III in the nucleolus and splicing speckles is an indicator of plant stress. *Plant Signal. Behav*. 4, 1148–1151.

Lamond, A.I., Spector, D.L. (2003) Nuclear speckles: a model for nuclear organelles. *Nat. Rev. Mol. Cell Biol*. 4, 605–612.

Leivar, P., Quail, P.H. (2011) PIFs: pivotal components in a cellular signaling hub. *Trends Plant. Sci*. 16, 19–28.

Li, B., Meng, X., Shan, L., He, P. (2016) Transcriptional regulation of pattern-triggered immunity in plants. *Cell Host Microbe*. 19, 641–650.

Li, F., Cheng, C., Cui, F., de Oliveira, M.V., Yu, X., Meng, X., Intorne, A.C., Babilonia, K., Li, M., Li, B. (2014) Modulation of RNA Polymerase II Phosphorylation Downstream of Pathogen Perception Orchestrates Plant Immunity. *Cell Host Microbe*. 16, 748–758.

Lorkovic, Z.J., Hilscher J., Barta, A. (2008) Co-localisation studies of *Arabidopsis* SR splicing factors reveal different types of speckles in plant cell nuclei. *Exp. Cell Res*. 314, 3175–3186.

Mao, Y.S., Zhang, B., Spector, D.L. (2011) Biogenesis and function of nuclear bodies. *Trends Genet.* 27, 295–306.

Okazawa, H., Rich, T., Chang, A., Lin, X., Waragai, M., Kajikawa, M., Enokido, Y., Komuro, A., Kato, S., Shibata, M., Hatanaka, H., Mouradian, M.M., Sudol, M., Kanazawa, I. (2002) Interaction between mutant ataxin-1 and PQBP-1 affects transcription and cell death. *Neuron*. 34, 701–713.

Padmanabhan, M.S., Ma, S., Burch-Smith, T.M., Czymmek, K., Huijser, P., Dinesh-Kumar, S.P. (2013) Novel Positive Regulatory Role for the SPL6 Transcription Factor in the N TIR-NB-LRR Receptor-Mediated Plant Innate Immunity. *PLoS Pathog*. 9, e1003235.

Papantonis, A., Cook, P.R. (2013) Transcription factories: genome organization and gene regulation. *Chem. Rev*. 113, 8683–8705.

Sawyer, I. A., Dundr, M. (2016) Nuclear bodies: Built to boost. *J. Cell Biol*. *213*, 509–511.

Shaw, P.J., Brown, J.W. (2004) Plant nuclear bodies. *Curr. Opin. Plant Biol*. 7, 614–620.

Tillemans, V., Dispa, L., Remacle, C., Collinge, M., Motte, P. (2005) Functional distribution and dynamics of *Arabidopsis* SR splicing factors in living plant cells. *Plant J*. 41, 567–582.

Tillemans, V., Leponce, I., Rausin, G., Dispa, L., Motte, P. (2006) Insights into nuclear organization in plants as revealed by the dynamic distribution of *Arabidopsis* SR splicing factors. *Plant Cell*. 18, 3218–3234.

van Zanten, M., Tessadori, F., Peeters, A.J., Fransz, P. (2012) Shedding light on large-scale chromatin reorganization in *Arabidopsis thaliana*. *Mol. Plant*. 5, 583–590.

Wang, G., Kong, H., Sun, Y., Zhang, X., Zhang, W., Altman, N., DePamphilis, C.W., Ma, H. (2004) Genome-wide analysis of the cyclin family in *Arabidopsis* and comparative phylogenetic analysis of plant cyclin-like proteins. *Plant Physiol*. 135,1084–1099.

Zhang, X., Henriques, R., Lin, S.S., Niu, Q.W., and Chua, N.H. (2006) Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral-dip method. *Nat. Protoc*. 1, 1–6.

Supplementary Information

Table S1 – The top ten gene ontology enriched categories in the *AtWWP1*-4 overexpressing line

GOMFID*	DESCRIPTION	SIZE	COUNT	FDR
GO:0008199	Ferric iron binding	5	4	0.000
GO:0016491	Oxidoreductase activity	1693	138	0.000
GO:0009055	Electron carrier activity	542	51	0.001
GO:0001047	Core promoter binding	4	3	0.001
GO:0003993	Acid phosphatase activity	45	9	0.001
GO:0003700	Sequence-specific DNA binding transcription factor activity	1683	129	0.001
GO:0004364	Glutathione transferase activity	48	9	0.002
GO:0050660	Flavin adenine dinucleotide binding	166	20	0.002
GO:0015294	Solute:cation symporter activity	126	16	0.003
GO:0008762	UDP-N-acetylmuramate dehydrogenase activity	43	8	0.003

*** GO Molecular function ID**

Table S2 – The top ten gene ontology enriched molecular function categories in *atwwp1* knockout line

GOMFID*	DESCRIPTION	SIZE	COUNT	FDR
GO:0003700	Sequence-specific DNA binding transcription factor activity	1683	225	0.000
GO:0003677	DNA binding	1571	171	0.000
GO:0016758	Transferase activity, transferring hexosyl groups	251	40	0.000
GO:1901363	Heterocyclic compound binding	6584	579	0.000
GO:0097159	Organic cyclic compound binding	6600	580	0.000
GO:0080043	Quercetin 3-O-glucosyltransferase activity	23	8	0.000
GO:0016740	Transferase activity	3307	301	0.000
GO:0008794	Arsenate reductase (glutaredoxin) activity	14	6	0.000
GO:0030613	Oxidoreductase activity, acting on phosphorus or arsenic in donors	14	6	0.000
GO:0004497	Monooxygenase activity	117	20	0.000

*** GO Molecular function ID**

Figures Legends

Figure 1 Subnuclear co-localization of AtWWP1 with nuclear body-associated protein markers

Fibrillarin for nucleolar pre-rRNA splicing, CCR2/ AtGRP7 for circadian rhythm, Coilin for Cajal Bodies, CypRS64 and SR34 for splicing, and CDKC2 for splicing and transcription were used as NB-associated markers. mCherry-fused nuclear body-associated markers and AtWWP1-GFP were expressed in combination in *N. benthamiana* leaves and the derived fluorescence observed by confocal microscopy. Scale bars, 10 μm.

Figure 2. AtWWP1 associates with CTD-RNAPII

(A) AtWWP1 interacts with CTD-RNAPII in yeast. AD-AtWWP1 and BD-CTD-RNAPII recombinant proteins were expressed in yeast and the interactions between the tested proteins were analyzed by monitoring His prototrophy on seletive medium.

(B) AtWWP1 and CTD-RNAPII interact in the nucleus of *N. benthamiana* cells. Cells expressing AtWWP1 and CTD-RNAPII fused to the YFP C-terminus (cYFP) or N-terminus (nYFP) were observed under the confocal microscope 3 days after infiltration of *Agrobacterium* carrying the respective DNA constructs. YFP fluorescence was observed in cells co-infiltrated with constructs corresponding to CTD-RNAPII-nYFP and AtWWP1-cYFP. Scale bars, 10 μm. DIC, differential interference contrast.

Figure 3. Sub-nuclear dynamics of AtWWP1-NB distribution

(A) The organization of AtWWP1-NBs is influenced by the level of transcription. Transgenic lines expressing AtWWP1-GFP were treated with the indicated chemicals for 3 h and the subnuclear distribution of AtWWP1-NB was examined by confocal microscopy. Scale bars, 5 μm.

(B) AtWWP1-NB does not co-localize with heterochromatin. The heterochromatin region was stained using DAPI.

Scale bars, 5μm

Figure 4. *In vitro* nucleic acid binding assay

(A) DNA binding assay using ssDNA- or dsDNA-attached cellulose beads. HA-fused proteins were transcribed and translated *in vitro* (input) and subsequently incubated with DNA-beads. Proteins were detected by western blot using anti-HA antibody. Histone H3 was used as a positive control.

(B) RNA binding assay using *A. thaliana* biotinylated RNA conjugated with streptavidin beads. *In vitro* expressed GST-fused candidates (input) were incubated with RNA-conjugated beads and subsequently analyzed by western blot using GST-antibody. AtGRP7 was used as a positive control and GST as a negative control.

Figure 5. AtWWP1 associates with AtMBD2 in the nucleus of plant cells and redistributes AtMBD2 to the AtWWP1-NBs

(A) Yeast two-hybrid assay of AtWWP1 and AtMBD2 interaction. Both proteins were expressed in yeast fused to either the activation domain (AD) and the binding domain (BD) of GAL4. The interactions between the tested proteins were analyzed by monitoring His prototrophy on seletive medium supplemented with 3AT. The full-length NIG fused to AD, which displays transactivation activity in yeast, was used as a positive control.

(B) Transactivation of the *β-galactosidase* reporter gene. The interactions were confirmed by measuring the activity of the β-galactosidase reporter enzyme activity

The transactivation capacity of NIG was monitored as a positive control. Asterisks indicate statistically significant differences comparing to the negative controls (t-test, $p \leq 0,05$, n=3).
(C) AtWWP1 interacts with AtMBD2 *in planta*. Protein extracts from *N. benthamiana* leaves transiently expressing AtWWP1-GFP and AtMBD2-HA fusions were used for co-imunoprecipitation assays. The Co-IP was performed using an anti-GFP antibody. Inputs controls show AtWWP1-GFP and AtMBD2-HA protein levels. Anti-haemagglutinin (HA) antibody was used to detect AtMBD2-HA from the immunoprecipitated complex.
(D) AtWWP1 and AtMBD2 interact in nuclear bodies. Constructs expressing AtWWP1 and AtMBD2 fused to the YFP C terminus (cYFP) or N terminus (nYFP) were observed under the confocal microscope 3 days after infiltration. DIC, differential interference contrast. Scale bars, 20 µm.
(E) Co-localization of AtMBD2 with AtWWP1 in nuclear bodies. Confocal image of subcellular localization of AtMBD2-mCherry transiently co-expressed with AtWWP1-GFP in N. *bethamiana* leaf cells. Scale bars, 10 µm.

Supplementary Figures Legends

Figure S1. *In vitro* DNA binding assay
DNA binding assay using ssDNA- or dsDNA-attached cellulose beads. HA-fused candidates (as indicated) were transcribed and translated *in vitro* (input) and subsequently incubated with DNA-beads. Proteins were detected by western blot using anti-HA antibody. PIF4 was used as a positive control and HA as a negative control.

Figure S2 AtWWP1 is connected to a transcription factor hub related to auxin signaling.
Schematic representation of AtWWP1 interaction network based on Arabidopsis Interactome database (http://signal.salk.edu/interactome/). AtWWP1 (dark green) is connected to an immune hub converging to CSN5A (blue) via NIG (yellow) interaction and it is also linked to auxin signaling-related transcription factors (light green) via AtMBD2 (brown) interaction. Red denotes begomovirus proteins that converge on CSN5A, yellow represents putative transcriptional factors of unknown functions, pink shows receptor-like kinases and gray indicates unknown proteins.

Figure S3. The C-terminal region of AtWWP1 mediates its interaction with AtMBD2
Yeast cells co-transformed with the indicated constructions were evaluated by monitoring His prototrophy on seletive medium supplemented with 3AT. The full-length NIG fused to AD, which displays transactivation activity in yeast, was used as a positive control.

Figures

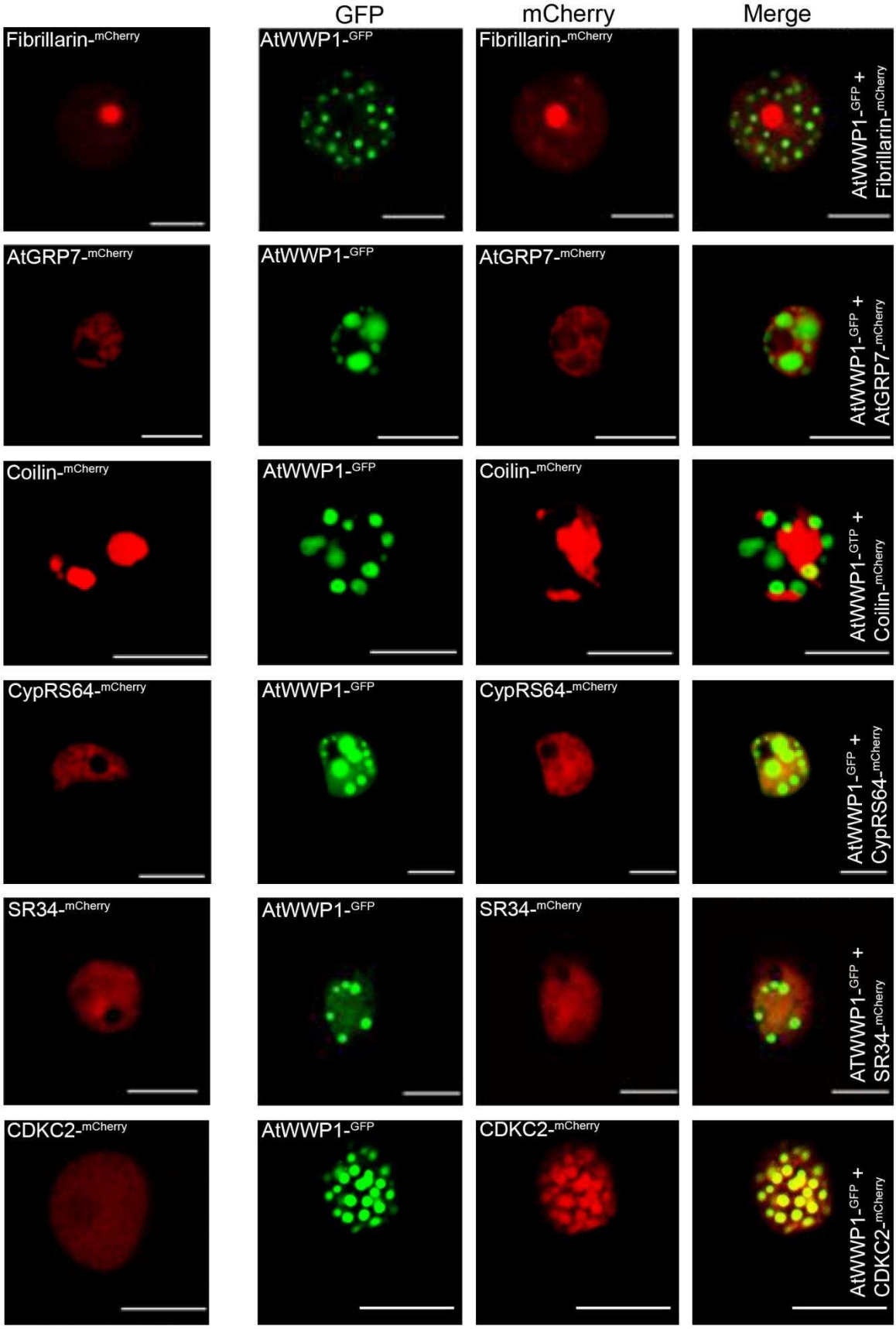
GFP
mCherry
Merge
Fibrillarin-mCherry
AtWWP1-GFP
Fibrillarin-mCherry
AtWWP1-GFP + Fibrillarin-mCherry
AtGRP7-mCherry
AtWWP1-GFP
AtGRP7-mCherry
AtWWP1-GFP + AtGRP7-mCherry
Coilin-mCherry
AtWWP1-GFP
Coilin-mCherry
AtWWP1-GTP + Coilin-mCherry
CypRS64-mCherry
AtWWP1-GFP
CypRS64-mCherry
AtWWP1-GFP + CypRS64-mCherry
SR34-mCherry
AtWWP1-GFP
SR34-mCherry
ATWWP1-GFP + SR34-mCherry
CDKC2-mCherry
AtWWP1-GFP
CDKC2-mCherry
AtWWP1-GFP + CDKC2-mCherry

Figure 1. Subnuclear co-localization of AtWWP1 with nuclear body-associated protein markers.
Fibrillarin for nucleolar pre-rRNA splicing, CCR2/ AtGRP7 for circadian rhythm, Coilin for Cajal Bodies, CypRS64 and SR34 for splicing, and CDKC2 for splicing and transcription were used as NB-associated markers. mCherry-fused nuclear body-associated markers and AtWWP1-GFP were expressed in combination in *N. benthamiana* leaves and the derived fluorescence observed by confocal microscopy. Scale bars, 10 μm

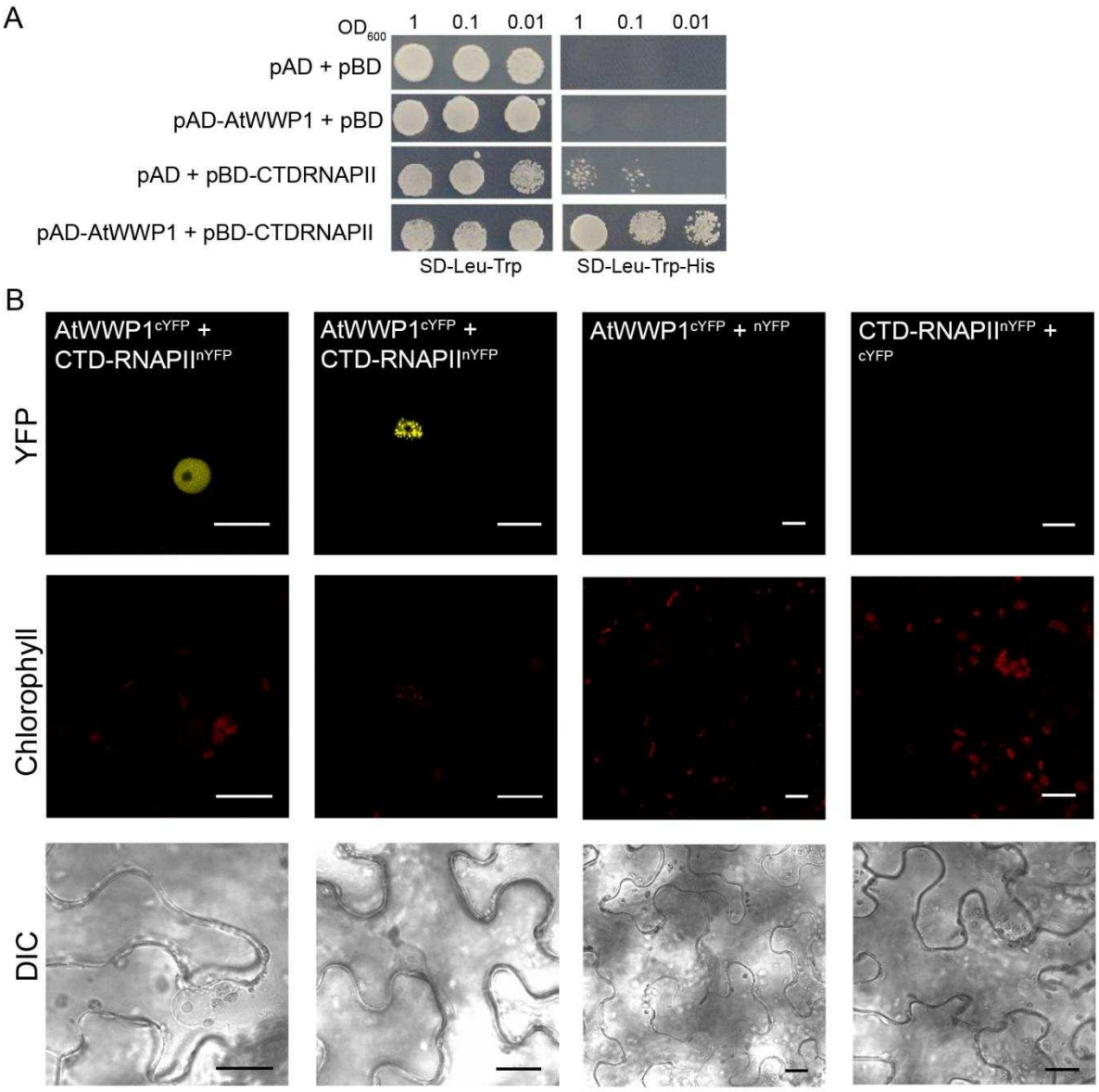

Figure 2. AtWWP1 associates with CTD-RNAPII
(A) AtWWP1 interacts with CTD-RNAPII in yeast. AD-AtWWP1 and BD- CTD-RNAPII recombinant proteins were expressed in yeast and the interactions between the tested proteins were analyzed by monitoring His prototrophy on seletive medium.
(B) AtWWP1 and CTD-RNAPII interact in the nucleus of *N. benthamiana* cells. Cells expressing AtWWP1 and CTD-RNAPII fused to the YFP C-terminus (cYFP) or N-terminus (nYFP) were observed under the confocal microscope 3 days after infiltration of *Agrobacterium* carrying the respective DNA constructs. YFP fluorescence was observed in cells co-infiltrated with constructs corresponding to CTD-RNAPII-nYFP and AtWWP1-cYFP. Scale bars, 10 µm. DIC, differential interference contrast.

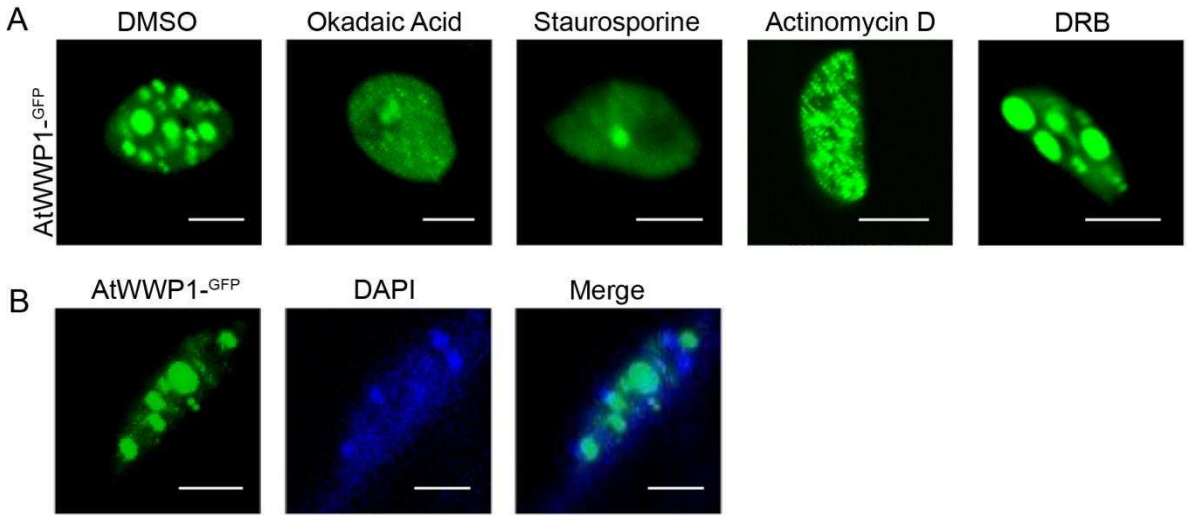

Figure 3. Sub-nuclear dynamics of AtWWP1-NB distribution
(A) The organization of AtWWP1-NBs is influenced by the level of transcription. Transgenic lines expressing AtWWP1-GFP were treated with the indicated chemicals for 3 h and the subnuclear distribution of AtWWP1-NB was examined by confocal microscopy. Scale bars, 5μm.
(B) AtWWP1-NB does not co-localize with heterochromatin. The heterochromatin region was stained using DAPI.Scale bars, 5μm.

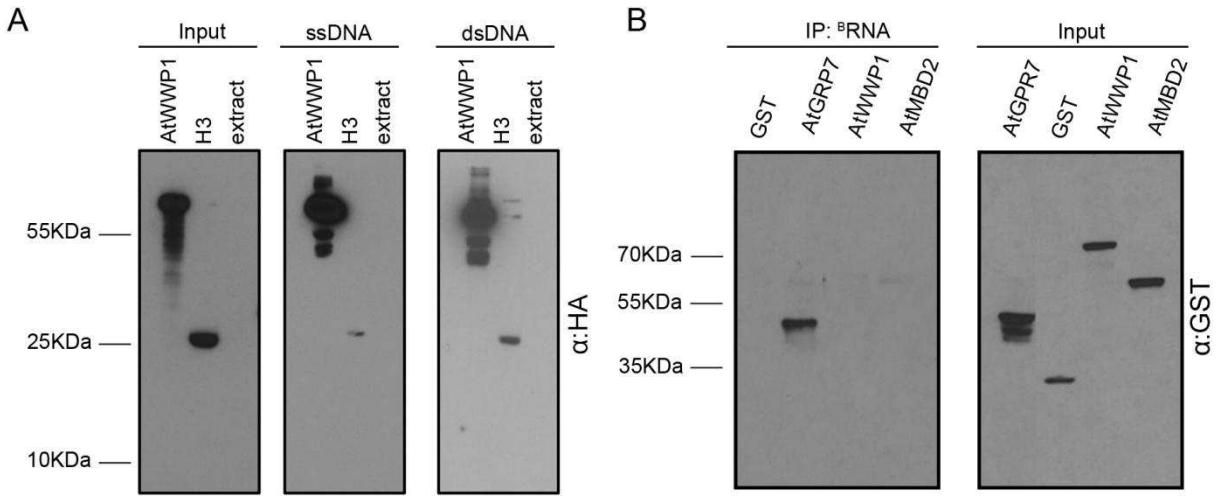

Figure 4. *In vitro* nucleic acid binding assay
(A) DNA binding assay using ssDNA- or dsDNA-attached cellulose beads. HA-fused proteins were transcribed and translated *in vitro* (input) and subsequently incubated with DNA-beads. Proteins were detected by western blot using anti-HA antibody. Histone H3 was used as a positive control.
(B) RNA binding assay using *A. thaliana* biotinylated RNA conjugated with streptavidin beads. *In vitro* expressed GST-fused candidates (input) were incubated with RNA-conjugated beads and subsequently analyzed by western blot using GST-antibody . AtGRP7 was used as a positive control and GST as a negative control.

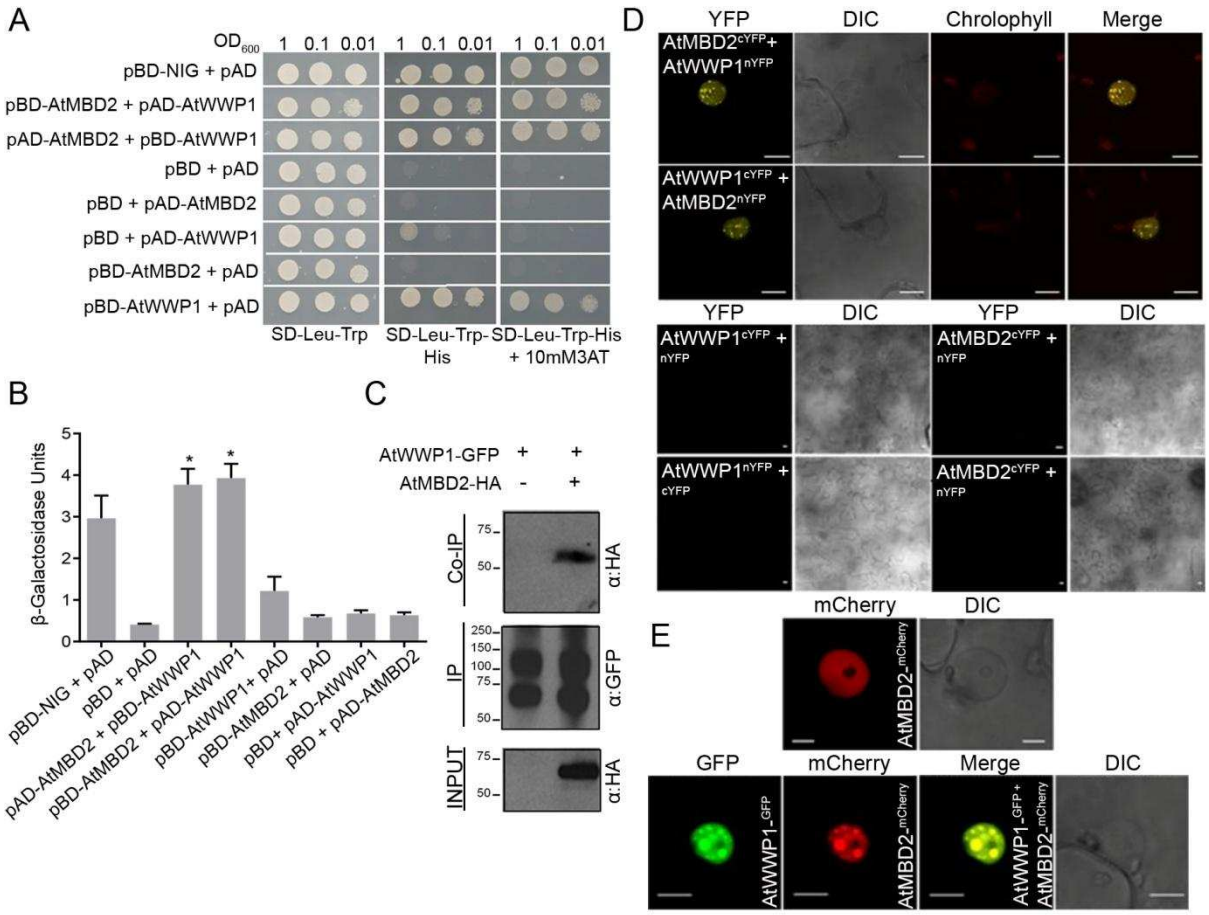

Figure 5. AtWWP1 associates with AtMBD2 in the nucleus of plant cells and redistributes AtMBD2 to the AtWWP1-NBs

(A) Yeast two-hybrid assay of AtWWP1 and AtMBD2 interaction. Both proteins were expressed in yeast fused to either the activation domain (AD) and the binding domain (BD) of GAL4. The interactions between the tested proteins were analyzed by monitoring His prototrophy on seletive medium supplemented with 3AT. The full-length NIG fused to AD, which displays transactivation activity in yeast, was used as a positive control.

(B) Transactivation of the *β-galactosidase* reporter gene. The interactions were confirmed by measuring the activity of the β-galactosidase reporter enzyme activity The transactivation capacity of NIG was monitored as a positive control. Asterisks indicate statistically significant differences comparing to the negative controls (t-test, $p \leq 0{,}05$, n=3).

(C) AtWWP1 interacts with AtMBD2 *in planta*. Protein extracts from *N. benthamiana* leaves transiently expressing AtWWP1-GFP and AtMBD2-HA fusions were used for co-imunoprecipitation assays. The Co-IP was performed using an anti-GFP antibody. Inputs controls show AtWWP1-GFP and AtMBD2-HA protein levels. Anti-haemagglutinin (HA) antibody was used to detect AtMBD2-HA from the immunoprecipitated complex.

(D) AtWWP1 and AtMBD2 interact in nuclear bodies. Constructs expressing AtWWP1 and AtMBD2 fused to the YFP C-terminus (cYFP) or N-terminus (nYFP) were observed under the confocal microscope 3 days after infiltration. DIC, differential interference contrast. Scale bars, 20 μm.

(E) Co-localization of AtMBD2 with AtWWP1 in nuclear bodies. Confocal image of subcellular localization of AtMBD2-mCherry transiently co-expressed with AtWWP1-GFP in N. *bethamiana* leaf cells. Scale bars, 10 μm.

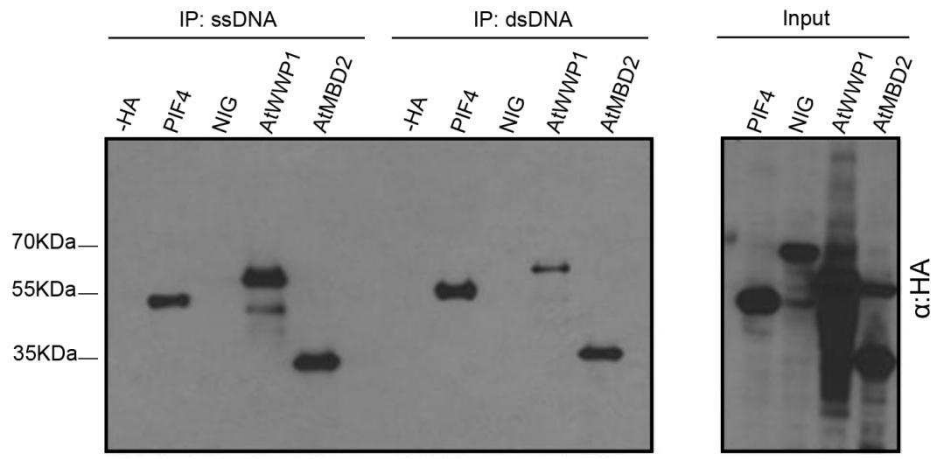

Figure S1. *In vitro* DNA binding assay
DNA binding assay using ssDNA- or dsDNA-attached cellulose beads. HA-fused candidates (as indicated) were transcribed and translated *in vitro* (input) and subsequently incubated with DNA-beads. Proteins were detected by western blot using anti-HA antibody. PIF4 was used as a positive control and HA as a negative control.

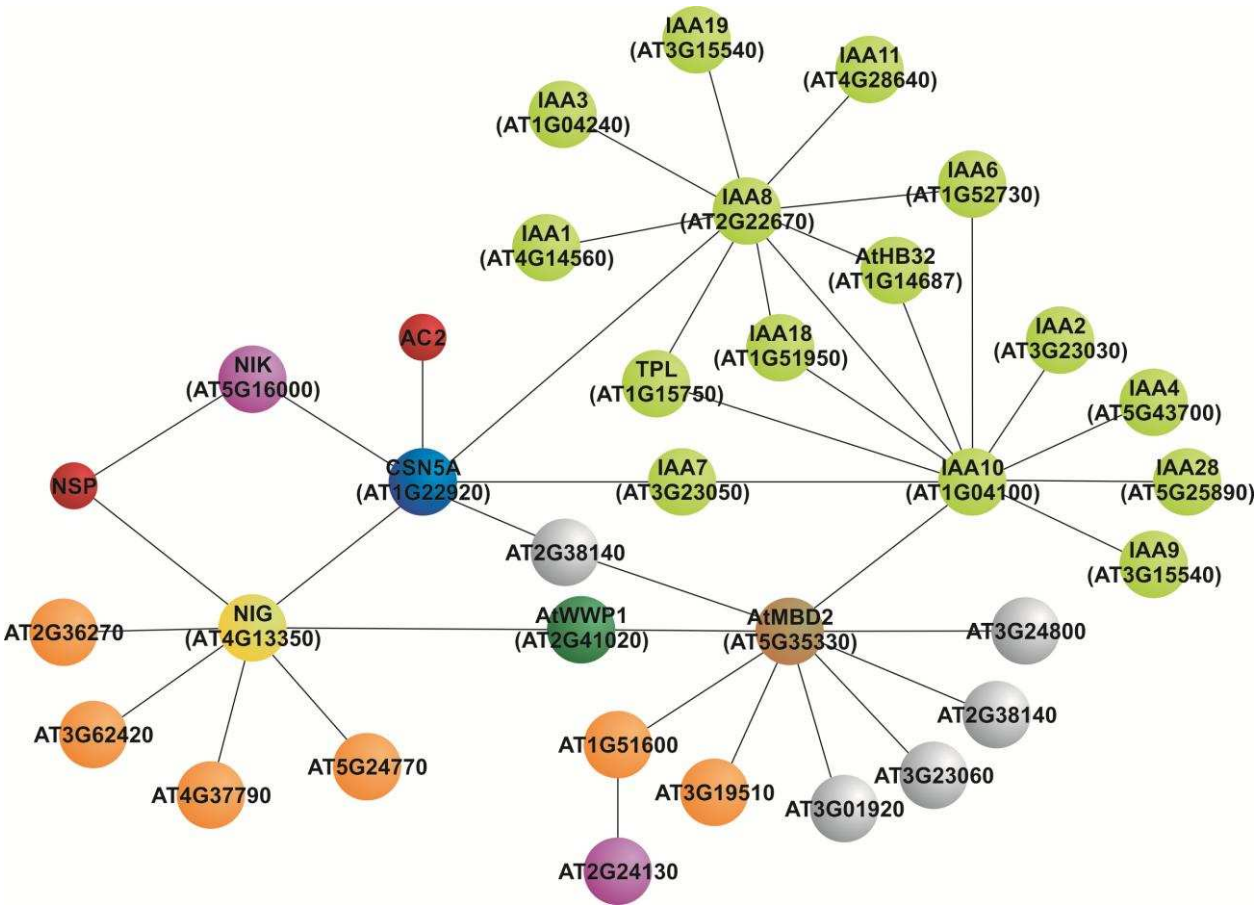

Figure S2 AtWWP1 is connected to a transcription factor hub related to auxin signaling

Schematic representation of AtWWP1 interaction network based on Arabidopsis Interactome database (http://signal.salk.edu/interactome/). AtWWP1 (dark green) is connected to an immune hub converging to CSN5A (blue) via NIG (yellow) interaction and it is also linked to auxin signaling-related transcription factors (light green) via AtMBD2 (brown) interaction. Red denotes begomovirus proteins that converge on CSN5A, yellow represents putative transcriptional factors of unknown functions, pink shows receptor-like kinases and gray indicates unknown proteins.

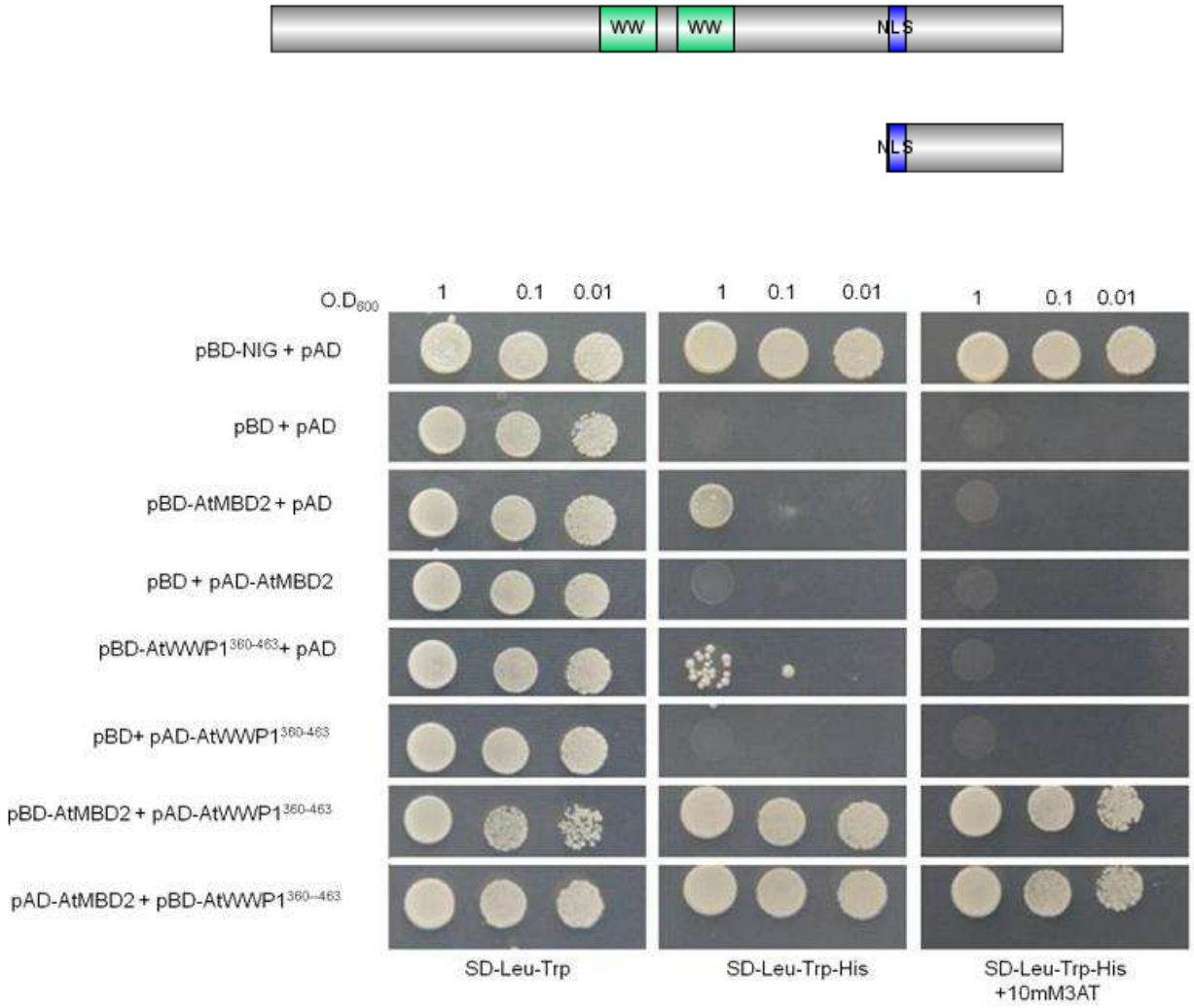

Figure S3. The C-terminal region of AtWWP1 mediates its interaction with AtMBD2

Yeast cells co-transformed with the indicated constructions were evaluated by monitoring His prototrophy on seletive medium supplemented with 3AT. The full-length NIG fused to AD, which displays transactivation activity in yeast, was used as a positive control.

GENERAL CONCLUSIONS

Among the environmental limitations that affect plant growth, viruses cause major crop losses and represent serious threat to food security. Successful infection by a plant virus depends on spreading their genomes between cells and throughout the organism. Thus, the identification of host factors involved in virus movement, direct or indirectly targeted by virus-encoded movement proteins, is crucial for the development of novel antiviral strategies. In this work, we reported the the characterization of AtWWP1, a WW domain-containing protein, identified as a NIG partner. We demonstrated that AtWWP1 forms nuclear bodies (NBs) via its WW domains and relocates NIG from the cytoplasm to the nucleus where it is confined into nuclear bodies. AtWWP1 display antiviral activity against begomoviruses, once loss of *AtWWP1* function debilitates further the plant upon infection and overexpression of *AtWWP1* confers tolerance to begomovirus. Consistently, viral infection interferes with the stability of AtWWP1 nuclear bodies. Additionally, AtWWP1 is dynamically integrated into a conserved immune hub as it interacts directly to CSN5A. We also provided evidence that AtWWP1 nuclear bodies are associated with active gene expression or co-transcriptional RNA processing. Our results have demonstrated that AtWWP1 is part of a plant antiviral immunity mechanism via nuclear inclusions. Further studies will help defining whether the antiviral role of AtWWP1 is linked to its nuclear bodies-forming activity.

ANEXOS

frontiers in Microbiology

REVIEW
published: 05 January 2017
doi: 10.3389/fmicb.2016.02139

Immune Receptors and Co-receptors in Antiviral Innate Immunity in Plants

Bianca C. Gouveia[1†], Iara P. Calil[1†], João Paulo B. Machado[1], Anésia A. Santos[2] and Elizabeth P. B. Fontes[1]*

[1] Department of Biochemistry and Molecular Biology, BIOAGRO, National Institute of Science and Technology in Plant-Pest Interactions, Universidade Federal de Viçosa, Viçosa, Brazil, [2] Department of General Biology, BIOAGRO, National Institute of Science and Technology in Plant-Pest Interactions, Universidade Federal de Viçosa, Viçosa, Brazil

Plants respond to pathogens using an innate immune system that is broadly divided into PTI (pathogen-associated molecular pattern- or PAMP-triggered immunity) and ETI (effector-triggered immunity). PTI is activated upon perception of PAMPs, conserved motifs derived from pathogens, by surface membrane-anchored pattern recognition receptors (PRRs). To overcome this first line of defense, pathogens release into plant cells effectors that inhibit PTI and activate effector-triggered susceptibility (ETS). Counteracting this virulence strategy, plant cells synthesize intracellular resistance (R) proteins, which specifically recognize pathogen effectors or avirulence (Avr) factors and activate ETI. These coevolving pathogen virulence strategies and plant resistance mechanisms illustrate evolutionary arms race between pathogen and host, which is integrated into the zigzag model of plant innate immunity. Although antiviral immune concepts have been initially excluded from the zigzag model, recent studies have provided several lines of evidence substantiating the notion that plants deploy the innate immune system to fight viruses in a manner similar to that used for non-viral pathogens. First, most R proteins against viruses so far characterized share structural similarity with antibacterial and antifungal R gene products and elicit typical ETI-based immune responses. Second, virus-derived PAMPs may activate PTI-like responses through immune co-receptors of plant PTI. Finally, and even more compelling, a viral Avr factor that triggers ETI in resistant genotypes has recently been shown to act as a suppressor of PTI, integrating plant viruses into the co-evolutionary model of host-pathogen interactions, the zigzag model. In this review, we summarize these important progresses, focusing on the potential significance of antiviral immune receptors and co-receptors in plant antiviral innate immunity. In light of the innate immune system, we also discuss a newly uncovered layer of antiviral defense that is specific to plant DNA viruses and relies on transmembrane receptor-mediated translational suppression for defense.

Keywords: resistance genes, receptor NIK1, PAMP-triggered immunity, effector-triggered immunity, antiviral immunity, ETI, PTI, NSP-Interacting kinase 1

OPEN ACCESS

Edited by:
Yule Liu,
Tsinghua University, China

Reviewed by:
Xiaorong Tao,
Nanjing Agricultural University, China
Magdalena Krzymowska,
Institute of Biochemistry and Biophysics (PAN), Poland

***Correspondence:**
Elizabeth P. B. Fontes
bbfontes@ufv.br

† These authors have contributed equally to this work.

Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *27 September 2016*
Accepted: *19 December 2016*
Published: *05 January 2017*

Citation:
Gouveia BC, Calil IP, Machado JPB, Santos AA and Fontes EPB (2017) Immune Receptors and Co-receptors in Antiviral Innate Immunity in Plants. Front. Microbiol. 7:2139. doi: 10.3389/fmicb.2016.02139

INTRODUCTION

Plants recognize potential pathogens mainly through two classes of distinct immune receptors (Schwessinger and Ronald, 2012; Spoel and Dong, 2012; Zvereva and Pooggin, 2012; Dangl et al., 2013). The first class consists of cell-surface associated pattern recognition receptors (PRRs), which are often represented by receptor-like kinases (RLKs) and receptor-like proteins (RLPs; **Figure 1**).

PRRs recognize conserved structural motifs present in microbes, which are known as microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs), or endogenous danger signals released by the plant during wounding or pathogenic attack, which are termed damage-associated molecular patterns (DAMPs; Macho and Zipfel, 2014). Perception of PAMPs by PRRs activates PAMP-triggered immunity (PTI), a transduction signal cascade that culminates with transcriptional reprograming and biosynthesis of specific defense molecules (Hogenhout et al., 2009; Bigeard et al., 2015). Activation of this immune response enables plants to respond rapidly and efficiently to a large range of pathogens (Roux et al., 2014). The second class of immune receptors includes intracellular immune receptors called R proteins (Jones and Dangl, 2006; Tsuda and Katagiri, 2010; **Figure 1**). These intracellular receptors directly or indirectly recognize effectors secreted by pathogens into the

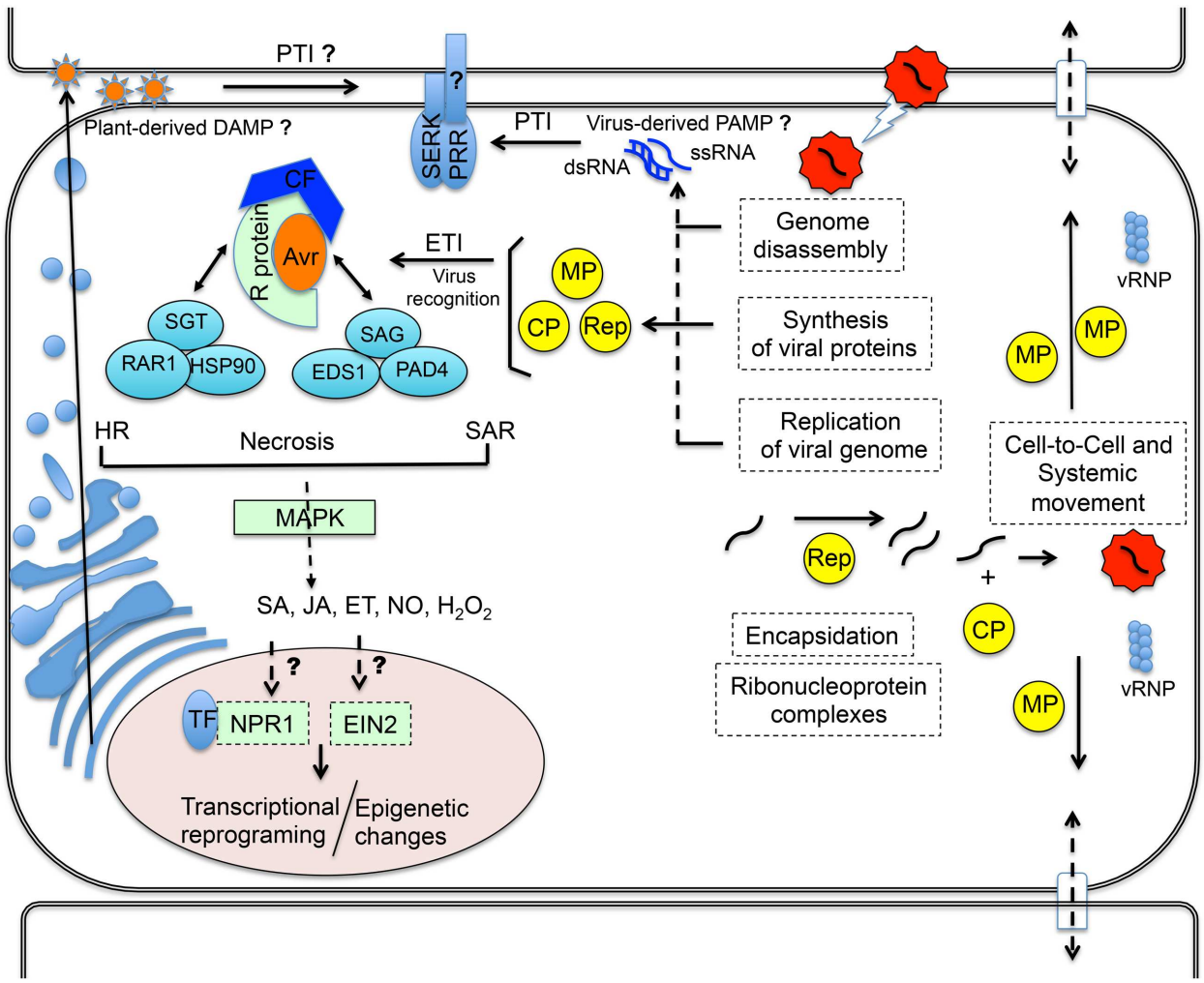

FIGURE 1 | Antiviral innate immunity with conserved features with antibacterial and antifungal immune responses. Plant viruses are obligate, biographic parasites and as such their life cycles start with the penetration of the virions in the host cells via wound sites (lightening arrow). Within the host cells, the virion is disassembled and then host cells mediate the expression of the viral genome by providing a translation apparatus for all viruses and transcription machinery for DNA viruses **(Figure 2)**. The viral mRNAs are translated into the cytoplasm, producing at least three viral proteins absolutely required for completion of the viral life cycle, replication protein (Rep), movement protein (MP) and coat protein (CP). The viral replication proteins combine with cellular proteins to produce multiple copies of the virus genome. These newly made genomes interact with CPs to form new virions or viral ribonucleoprotein complexes (vRNP). The next step is movement of the virus into neighboring cells, which requires the MP. The intracellular translated viral proteins (Avr) may also provide recognition sites for cytosolic NB-LRR receptors (e.g., R proteins), triggering ETI, which results in HR, necrosis or SAR similarly to non-viral ETI. R proteins, R co-factors (CF) and Avr factors form an interacting complex with the SGT1/RAR1/HSP90, and EDS1/PAD4/SAG101 modules to mediate downstream changes in SA, JA, ET, NO and H_2O_2 levels or signaling via MAP Kinases cascades, culminating in the induction of defense genes. NPR1 complexes TF to induce defense genes via SA signaling, whereas EIN2 is a regulator of ET signaling. Virus infection may also trigger epigenetic changes. At the first line of defense, replication of viral RNA genomes may provide non-self RNA motifs (ssRNA or dsRNA) as virus-derived PAMPs to activate PTI. Alternatively, plant cells may sense viral infection and secrete plant-derived DAMPs, recognized by PRRs extracellularly. Members of the SERK family also function as co-receptors in viral PTI. Arrows denote unknown or putative paradigms in viral innate immunity. Adapted from Mandadi and Scholthof (2013).

host intracellular environment and activate effector-triggered immunity (ETI; Howden and Huitema, 2012), which is often manifested in the hypersensitive response (HR) associated with rapid cell death, production of reactive oxygen species (ROS) and salicylic acid (SA) as well as expression of defense-related genes (Win et al., 2012). This is considered to be a more robust defense compared to PTI (Coll et al., 2011; Reimer-Michalski and Conrath, 2016). The effectors that are specifically detected by matching R proteins to activate ETI are termed avirulence (Avr) proteins. Pathogens containing Avr genes are avirulent to plants carrying the cognate R genes and virulent to plants without the R genes. Due to the limitation of the coding capacity of viral genomes, virtually all virus proteins, such as replicase, movement proteins (MPs), coat proteins (CPs), can act as Avr determinants. Therefore, virus Avr proteins are usually necessary for successful infection and are almost invariably virulence factors in a susceptible host.

Studies in plant–virus interactions have pioneered the description of paradigms in plant immune response, including the HR and systemic acquired resistance (SAR; Holmes, 1929, 1938; Ross, 1961). Nevertheless, current semantics and concepts regarding plant immunity models were built to fill the findings on bacterial and fungal infections and hence antiviral immune concepts were initially excluded from these models (Jones and Dangl, 2006; Bent and Mackey, 2007; Boller and Felix, 2009; Dodds and Rathjen, 2010; Schwessinger and Ronald, 2012; Spoel and Dong, 2012). Recently, Mandadi and Scholthof (2013) proposed reconciling the differences and perpetuating the analogy between antiviral and anti-non-viral immune concepts into definitions of viral effectors, viral ETI and viral PTI. These definitions, as described below, integrate antiviral immune concepts into current plant immunity models.

Typical bacterial and fungal effectors are delivered into host cells via microbial secretion systems, whereas viral effectors encoded by the viral genome are directly translated into the host cytoplasm. These factors share similar functions because bacterial and fungal effectors interfere with PTI or other immune regulators and viral effectors promote virulence by interfering with host defense pathways. Although not covered in this review, viral suppressors of RNA silencing are also included in this category. Similar to non-viral pathogen effectors, in resistant genotypes, the intracellularly translated viral effectors are recognized by R proteins, triggering immune responses that often are associated with hallmarks of ETI, such as HR, SA accumulation, ROS production and SAR. Therefore, virus Avr factors, which interfere with defenses, are also referred to as viral effectors, and the immune response they trigger is also referred to as ETI. However, viral ETI is independent with regard to the nature of the immune response, which may or may not be associated with hallmarks of bacterial or fungal ETI. The notion that viruses encode PAMPs recognized by PRRs, such as virus-derived nucleic acids, is well documented in animal systems, and recent evidence has extended the concept of viral PTI to plant–virus interaction systems.

An additional recently uncovered virus-specific defense mechanism relies on suppression of host translation mediated by the transmembrane immune receptor NUCLEAR SHUTTLE PROTEIN-INTERACTING KINASE 1 (NIK1), which was first identified as a virulence target of begomovirus NSP (**Figure 2**). Activation of NIK1-mediated antiviral signaling leads to translocation of the ribosomal protein L10 (RPL10) to the nucleus, where it interacts with L10-INTERACTING MYB DOMAIN-CONTAINING PROTEIN (LIMYB) to fully repress expression of translational machinery-related genes and global host translation. Begomovirus mRNAs are unable to escape this translational regulatory mechanism of plant cells and hence are not efficiently translated, which compromises infection upon activation of NIK1-mediated defense. Although the NIK1-mediated defense response is remarkably dissimilar from the PTI response, structural components and activation of the NIK1 immune receptor as well as its interaction with virus infection exhibit features reminiscent of the plant innate immunity mechanism.

This review focuses on the concepts of viral ETI and viral PTI, describing antiviral immune receptors and co-receptors involved in antiviral innate immunity in plants. Furthermore, we describe NIK1-mediated antiviral signaling, a newly discovered layer of antiviral defense, which is specific to plant DNA viruses and relies on transmembrane receptor-mediated translational suppression for defense. This latter level of antiviral defense is discussed within the context of the innate immune system.

EFFECTOR-TRIGGERED IMMUNITY IN ANTIVIRAL DEFENSE: R GENE-MEDIATED RESPONSES TO VIRUS INFECTION

Activation of ETI, involving strain-specific recognition of a virus-encoded effector through direct or indirect interaction with a corresponding resistance gene (R gene) product, can lead to the hypersensitive reaction (HR). HR is considered a resistance response against several different pathogens that, to the some extent, occurs through similar mechanisms. Similar to non-viral infections, the HR response during viral infection is initiated by direct or indirect Avr-R interactions and is frequently associated with accumulation of SA in both infected and non-infected tissues (Culver and Padmanabhan, 2007; Carr et al., 2010; Pallas and García, 2011; Mandadi and Scholthof, 2012). HR is also associated with perturbation in Ca^{++} homeostasis, membrane integrity and activation of caspase-like proteases, such as the vacuolar processing enzyme that is considered an executioner of cell death during HR (Mur et al., 2008). Although cell death is often associated with HR-mediated resistance, HR may be uncoupled from resistance, an interpretation that arises from compelling biochemical and genetic studies of *Potato virus X* (PVX), *Tomato bushy stunt virus* (TBSV), *Cauliflower mosaic virus* (CaMV) and *Tomato mosaic virus* (ToMV; Bendahmane et al., 1999; Chu et al., 2000; Cole et al., 2001; Ishibashi et al., 2007, 2009). For instance, the tomato resistance protein Tm-1 relays resistance against ToMV by inactivating the ToMV replicase protein

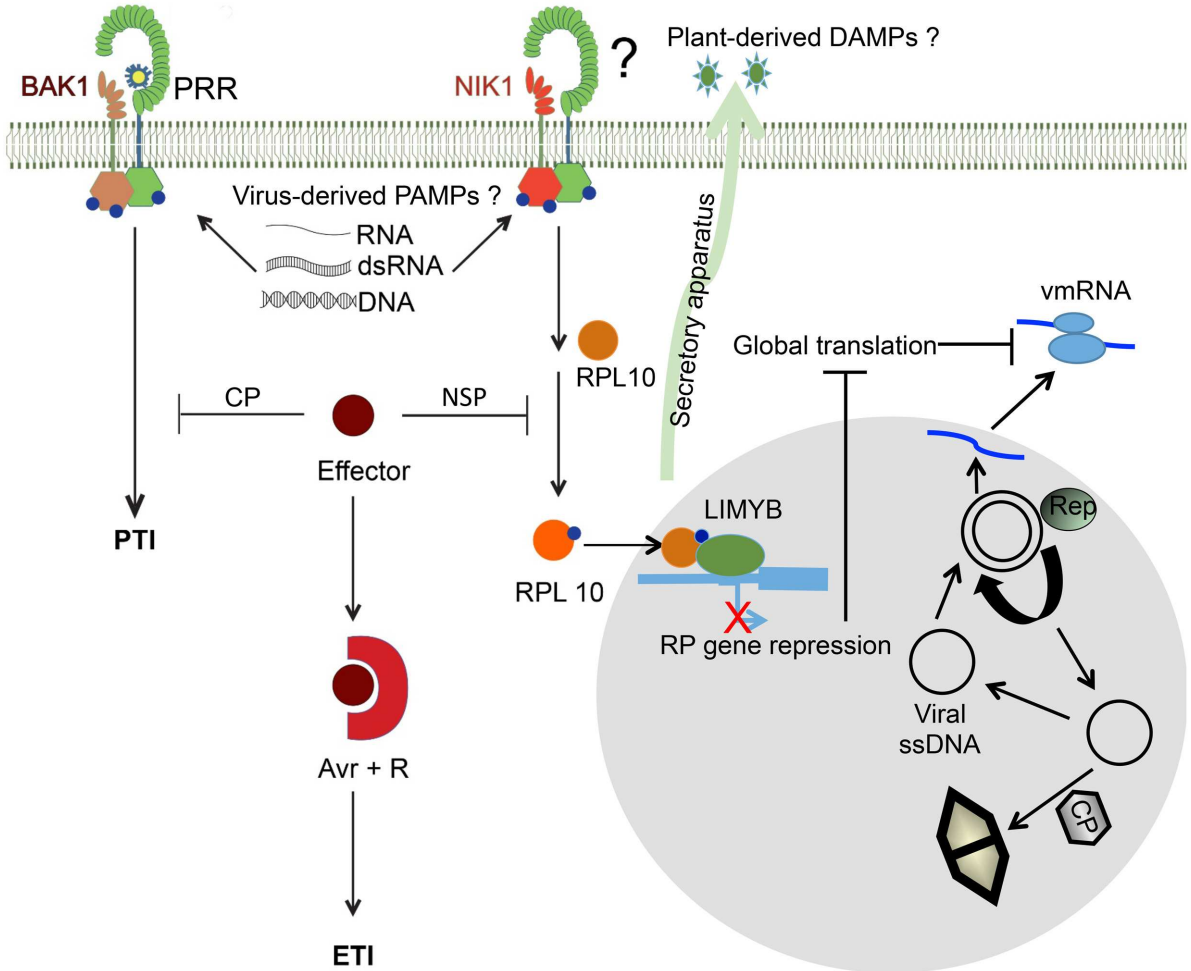

FIGURE 2 | Similarities between viral PTI and NIK1-mediated antiviral signaling. Replication and expression of viral genomes lead to the accumulation of non-self DNA or RNA motifs (virus-derived PAMPs), which may be recognized by PRRs that in turn heteromultimerize with co-receptors (BAK1 or SERK1) to trigger viral PTI. Alternatively, PTI may be activated by endogenous DAMPs, which are induced by virus infection and delivered to the apoplast via the secretory apparatus. In addition to PTI, in the case of DNA viruses (begomoviruses), plant cells may also elicit the translational control branch of the NIK1-mediated antiviral signaling as an innate defense. The mechanism of NIK1 transmembrane receptor activation is unknown. Structural organization and biochemical properties of NIK1 may suggest an activation mechanism dependent on recognition of viral PAMPs or endogenous DAMPs by PRR partners, similarly to a typical viral PTI. In this case, one may consider virus derived-dsDNA as possible PAMPs. The viral single-stranded DNA form begomoviruses replicates via double-stranded DNA intermediates that are transcribed in the nucleus of plant-infected cells. NSP binds to the nascent viral DNA and facilitates its movement to the cytoplasm and acts in concert with the classical MP to transport the viral DNA to the adjacent, uninfected cells. Activation of NIK1 in incompatible interactions promotes phosphorylation and subsequent translocation of RPL10 to the nucleus, where it interacts with LIMYB to fully repress the expression of RP genes, leading to global translation suppression, which also impairs viral mRNA (vmRNA) translation. In begomovirus-host compatible interactions, NSP binds to NIK1 and suppresses its activity. In any case, RNA or DNA viruses, a successful infection implicates in accumulation of virus effectors (for example, CP from PPV and NSP from begomoviruses) to suppress PTI, leading to disease. In resistant genotypes, however, the resistance genes specifically recognize, directly or indirectly, the viral effectors, called avirulence (Avr) factors, activating ETI and conferring resistance. Adapted from Machado et al. (2015).

without eliciting HR-associated cell death (Ishibashi et al., 2007, 2009).

As for non-viral pathogens, most plant antiviral R genes encode NB-LRR [nucleotide-binding-leucine-rich repeat (LRR)] proteins that mediate resistance via specific (direct or indirect) recognition of a virus Avr factor (Win et al., 2012) (**Table 1**). Based on their variable N-terminal domain, these plant NB-LRR proteins are further classified into coiled-coil (CC)-NB-LRR or Toll/interleukin 1 receptor-like (TIR)-NB-LRR protein families (Bonardi et al., 2012). Most of the known antiviral R proteins are CC-NB-LRR-like, whereas only a small number belong to the TIR-NB-LRR class (Zvereva and Pooggin, 2012; de Ronde et al., 2014). Recognition of effectors by R proteins may occur through direct ligand-receptor interactions (gene-for-gene model; Flor, 1971) or through indirect interactions (Guard Model; Jones and Dangl, 2006; Oßwald et al., 2014). In the Guard Model, the resistance protein guards a target host protein, the guardee, and perceives alterations in this target protein upon interaction

TABLE 1 | Plant antiviral NB-LRR resistance genes and the cognate avirulence determinants.

Gene	Plant	R protein signature	Virus	Avr factor	Reference
N	*Nicotiana glutinosa*	TIR-NB-LRR	Tobacco mosaic virus (TMV)	Replicase	Whitham et al., 1994; Padgett et al., 1997
Rx1	*Solanum tuberosum*	CC-NB-LRR	Potato virus *X* (PVX)	Coat Protein	Bendahmane et al., 1999
Rx2	*S. tuberosum*	CC-NB-LRR	PVX	Coat Protein	Bendahmane et al., 2000
HRT	*Arabidopsis thaliana* ecotype Dijon-17	CC-NB-LRR	Turnip crinkle virus (TCV)	Coat Protein	Cooley et al., 2000; Ren et al., 2000
RCY1	*A. thaliana* ecotype C24	CC-NB-LRR	Cucumber mosaic virus strain y	Coat Protein	Takahashi et al., 2001, 2002
Sw-5	Solanum peruvianum	SD-CC-NB-LRR	Tomato spotted wilt virus	Movement protein (NS)	Brommonschenkel et al., 2000; Spassova et al., 2001; Hallwass et al., 2014; Peiro et al., 2014
Y-1	*S. tuberosum*	TIR-NB-LRR	Potato virus *Y*	?	Vidal et al., 2002
Tm-22	*Solanum lycopersicum*	CC-NB-LRR	Tomato mosaic virus (ToMV)	Movement protein	Lanfermeijer et al., 2003
BcTuR3	*Brassica campestris*	TIR-NB-LRR	Turnip mosaic virus	?	Ma et al., 2010
Rsv1	*Glycine max*	CC-NB-LRR	Soybean mosaic virus	P3 + HC-Pro	Hayes et al., 2004; Wen et al., 2013
Pv1	*Cucumis melo*	TIR-NB-LRR	Papaya ringspot virus	?	Anagnostou et al., 2000
Pv2	*Cucumis melo*	TIR-NB-LRR	Papaya ringspot virus	?	Brotman et al., 2013
Cv (locus)	*Poncirus trifoliata*	CC-NB-LRR	Citrus tristeza virus	?	Yang et al., 2003
CYR1	*Vigna mungo*	CC-NB-LRR	Mungbean yellow mosaic virus	Coat Protein	Maiti et al., 2012
Pvr4	*Capsicum annuum*	CC-NB-LRR	Potato virus *Y* Pepper mottle virus	RNA-dependent RNA polymerase (NIb)	Kim et al., 2015, 2016
Tsw	*Capsicum chinense*	CC-NB-LRR	Tomato spotted wilt virus	NSs RNA silencing suppressor	Margaria et al., 2007; Ronde et al., 2013, 2014; Kim et al., 2016

Avr, avirulence; CC, coiled- coil; NB, nucleotide binding; LRR, leucine-rich repeat; SD, solanaceous-specific domain; TIR, Toll/interleukin 1 receptor-like.

with the pathogen effectors. Therefore, the modification of the guardee by the effector causes activation of the R protein to initiate a resistance response. Implicit in the Guard Model is the notion that the guarded effector target is indispensable for the virulence function of the effector protein in the absence of the cognate R protein (Dangl and Jones, 2001; Jones and Dangl, 2006). Alternatively, in the Decoy Model, a decoy (effector target mimic) evolved to act as a molecular sensor to only detect a pathogen without having any other role in the basic cellular machinery of the host (Van der Hoorn and Kamoun, 2008). Therefore, effector alteration of the decoy triggers innate immunity in plants that carry the cognate R protein but does not result in enhanced pathogen fitness in plants that lack the R protein.

The R signaling cascade in plant–virus interactions consists of rapid activation of MAP kinases and involvement of molecular chaperone complexes controlling R protein stabilization and destabilization (Kadota and Shirasu, 2012; Hoser et al., 2013). Convergence between viral and non-viral ETI is observed at the chaperone protein complex containing HEAT SHOCK PROTEIN 90 (HSP90), SUPPRESSOR OF THE G2 ALLELE OF SKP1 (SGT1) and REQUIRED FOR MLA12 RESISTANCE1 (RAR1). The HSP90/RAR1/SGT1 chaperone complex contributes to the stability and proper folding of R proteins during activation, mediating downstream MAP kinase activation, changes in defense gene expression and hormone levels (Liu et al., 2004; Dodds and Rathjen, 2010). Examples of R proteins against viruses that use the HSP90/RAR1/SGT1signaling module to mediate antiviral resistance are the N protein and Rx protein, which confer resistance against Tobacco mosaic virus (TMV) and PVX, respectively (**Table 1**) (Liu et al., 2004; Botër et al., 2007). Another functional module comprising ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1; Aarts et al., 1998; Falk et al., 1999), PHYTOALEXIN DEFICIENT4 (PAD4; Feys et al., 2001, 2005) and SENESCENCE-ASSOCIATED GENE101 (SAG101) mediates HR against viral and non-viral pathogens in a similar manner. In Arabidopsis, the EDS1/PAD4/SAG101 complex regulates HRT-mediated resistance against *Turnip crinkle virus* (TCV; **Table 1**) (Zhu et al., 2011). The HRT-mediated resistance also requires a functional SA-mediated signaling pathway (Chandra-Shekara et al., 2004). Disruption of SA signaling compromises HRT-mediated resistance without affecting HRT-mediated HR, providing further evidence that HR and resistance, albeit closely related, are unlinked processes. Therefore, virus-triggered ETI responses also involve functional SGT1/RAR1/HSP90 (Liu et al., 2004) and EDS1/PAD4/SAG101 (Zhu et al., 2011) protein complexes.

The tobacco *N* gene (for necrotic-type response), which confers resistance against TMV and encodes a TIR-NB-LRR

protein, was the first identified R gene (Holmes, 1938; Whitham et al., 1994). TMV is a positive-sense single-stranded RNA virus of 6.3–6.5 kb that encodes at least four proteins (Goelet et al., 1982; Osman and Buck, 1996). They include a 126-kDa replicase (with methyltransferase and RNA helicase domains), which is encoded by the 5′ORF of TMV and is directly translated from genomic RNA; the stop codon of which is read through to give a 183-KDa RNA-dependent RNA polymerase (RDR). The other two viral proteins, a MP and a capsid protein (CP), are expressed from separate subgenomic RNAs. The N resistance protein directly interacts with the helicase domain (the p50 effector) of TMV replicase to trigger resistance (Ueda et al., 2006). In fact, ectopic expression of the C-terminal 50 kDa portion (p50) of the 126 kDa replicase is sufficient to induce HR in tobacco carrying the *N* gene (Erickson et al., 1999). Full resistance to TMV, however, depends on N receptor-interacting protein 1 (NRIP1), which is recruited from chloroplasts to the cytoplasm and nucleus by the p50 effector and interacts directly with the N resistance protein (Caplan et al., 2008). The nuclear localization of the N resistance protein, which has been demonstrated to be critical for N-mediated resistance, is controlled by upstream events of receptor activation (Burch-Smith et al., 2007; Hoser et al., 2013). As a plant NB-LRR, the N protein requires the conserved chaperone complex consisting of HSP90, RAR1 and SGT for proper folding, accumulation and regulation (Liu et al., 2004). The assembly of this chaperone complex with the N protein occurs in the cytoplasm and SGT controls the nucleocytoplasmic partitioning of the immune receptor (Hoser et al., 2013). Upon TMV infection, p50 binds first to the TIR domain and then to the NB and LRR domains of the N protein leading to conformational changes and oligomerization of the immune receptor (Mestre and Baulcombe, 2006). Phosphorylation of SGT1 by an activated SIPK, a tobacco MAPK6 homolog, shifts the balance toward its nuclear distribution and consequently the N receptor complex is distributed to the nucleus (Burch-Smith et al., 2007; Hoser et al., 2013). Within the nucleus, N protein interacts with transcriptional factors (TFs) to modulate the expression of defense-related genes. The SQUAMOSA PROMOTER BINDING PROTEIN (SBP)-domain transcription factor SPL6 is an example of TF that interacts with the N immune receptor and positively regulates a subset of defense genes (Padmanabhan et al., 2013). This association is detected only when the TMV effector, p50, is present in the cell and is required for N-mediated resistance. SPL6 from Arabidopsis also functions in resistance against the bacterial pathogen *Pseudomonas syringae* expressing the AvrRps4 effector, as SPL6 is required for the R protein RPS4-mediated resistance (Padmanabhan et al., 2013). Therefore, the SPL6-mediated modulation of defense gene expression represents another convergent point in R-mediated resistance against both viruses and bacteria.

The *Rx* gene in potato encodes a well-characterized representative of the CC-NB-LRR class of R proteins, which mediates extreme resistance against PVX elicited by the viral CP. PVX is also a monopartite positive-sense single-stranded RNA virus (Huisman et al., 1988). Unlike other disease resistance responses, this extreme resistance is not associated with HR at the site of infection but rather is associated with an early arrest of viral accumulation in single cells (Bendahmane et al., 1999). The Rx protein also associates with the molecular chaperone HSP90 and its signaling proteins SGT1 and RAR1 to modulate the innate immune response in plants (Botër et al., 2007). The cochaperone SGT1 also interferes with the nucleocytoplasmic distribution of Rx protein (Slootweg et al., 2010; Hoser et al., 2014). Accordingly, silencing the cochaperone SGT1 impaired the accumulation of Rx1 protein in the nucleus and Rx distribution exactly mirrored that of ectopic AtSGT1b variants with forced cytoplasmic or nuclear localization. The Rx nucleocytoplasmic partitioning is also controlled by the Rx interacting partner RanGAP2 (Tameling et al., 2010). The Rx N-terminal CC domain interacts intramolecularly with the Rx NB-LRR region and intermolecularly with the Rx cofactor RanGAP2 (Ran GTPase-activating protein 2; Rairdan et al., 2008; Tameling et al., 2010). In fact, the crystal structure of the CC domain of Rx in complex with the Trp-Pro-Pro (WPP) domain of RanGAP2 reveals that the Rx CC domain forms a heterodimer with RanGAP2, which may prevent Rx self-association (Hao et al., 2013). The C-terminus of the LRR domain is thought to be involved in specific recognition of the viral effector, CP, although direct interaction between CP and Rx has not been demonstrated (Bendahmane et al., 1995; Dangl and Jones, 2001; Farnham and Baulcombe, 2006; Candresse et al., 2010). The Rx-interacting protein RanGAP2 controls Rx nucleocytoplasmic distribution and can act as a cytoplasmic retention factor for Rx. CP of PVX is recognized in the cytosol, and signaling is also activated in this compartment. Concentrating Rx in the cytosol via RanGAP2 overexpression enhances resistance signaling, whereas sequestering Rx in the nucleus through interaction with a nuclear-localized version of RanGAP2 inhibits resistance signaling (Slootweg et al., 2010; Tameling et al., 2010). However, nuclear export signal-mediated expulsion of Rx from the nucleus moderately reduced resistance, indicating that the nuclear pool of Rx also functions in immunity. These results demonstrate that both nuclear and cytoplasmic pools of NB-LRR Rx1 are necessary for full immune responses to PVX. Therefore, in both Rx-mediated resistance and N-mediated resistance, the R protein is activated in the cytoplasm, yet full functionality of the Rx and N R proteins depends on their nucleocytoplasmic distribution.

A few dominant resistance genes encoding the non-NB-LRR class of proteins have been characterized; these proteins have been found to function as sensors of virus infection but do not induce typical ETI-like defense responses, such as HR (**Table 2**). One such example is the tomato *Tm-1* gene, which confers dominant resistance to ToMV and contains two conserved domains: an uncharacterized N-terminal region (residues M1–K431) and a TIM-barrel-like C-terminal domain (residues T484–E754; Ishibashi et al., 2012, 2014; Yang et al., 2016). Tm-1 binds to ToMV replication proteins and inhibits ToMV multiplication without inducing a defense response: binding of Tm-1 to ToMV replication proteins inhibits the RNA-dependent RNA replication of ToMV and replication complex assembly on membranes that

TABLE 2 | Plant antiviral non-NB-LRR resistance genes and the cognate avirulence determinants.

Gene	Plant	R protein signature	Virus	Avr determinant?	Reference
JAX1	*Arabidopsis thaliana*	Jacalin-like [lectin gene]	Broad resistance against potexvirus	?	Yamaji et al., 2012
RTM1	*Arabidopsis thaliana*	Jacalin-like	Tobacco etch virus	Coat Protein	Chisholm et al., 2000
RTM2	*Arabidopsis thaliana*	Jacalin-like	Plum pox virus	Coat Protein	Whitham et al., 2000; Decroocq et al., 2009
Ty-1, Ty-3	*Solanum chilense*	RDR	Tomato yellow leaf curl virus	?	Verlaan et al., 2013; Butterbach et al., 2014
Tm-1	*Solanum hirsutum*	TIM-barrel-like domain protein	ToMV	Replicase, Helicase domain	Ishibashi et al., 2007; Kato et al., 2013

Avr, avirulence; RDR, RNA-dependent RNA polymerase.

precedes negative-strand RNA synthesis (Ishibashi and Ishikawa, 2013, 2014). Another recently characterized example of non-NB-LRR R proteins is the sensor proteins Ty-1 and Ty-3, which confer resistance to *Tomato yellow Leaf Curl Virus* (TYLCV). The *Ty-1* and *Ty-3* genes are allelic and code for an RDR of the RDRc type, which has an atypical DFDGD motif in the catalytic domain (Verlaan et al., 2013). The mechanism of resistance is completely uncoupled from ETI and appears to be linked to the RNA silencing strategy of antiviral defense (Butterbach et al., 2014). Accordingly, *Ty-1/Ty-3* plants display enhanced siRNA levels that coincide with hypermethylation of the TYLCV V1 (CP) promoter, indicating that *Ty-1*-based resistance against TYLCV involves enhanced transcriptional gene silencing.

In summary, most antiviral dominant resistance genes so far characterized encode typical NB-LRR R proteins (**Table 1**), which specifically recognize viral effectors or Avr factors and utilize signaling modules such as SGT1/RAR1/HSP90 and EDS1/PAD4/SAG101 complexes to mediate resistance responses, similar to non-viral pathogens. Therefore, plants appear to have evolved strategies and signaling modules to defend themselves against a large spectrum of pathogen types, such as bacteria, viruses and fungi. This interpretation allows us to integrate some aspects of the antiviral immune concepts into the typical bacterial and fungal immunity models to classify viral effectors and viral ETI.

PAMP-TRIGGERED IMMUNITY IN ANTIVIRAL DEFENSES: CO-RECEPTORS PAVE THE WAY

Plant innate defense responses are also activated upon perception of conserved PAMPs, which are pathogen-derived conserved motifs. In addition, endogenous molecules released by the host during pathogenic attack or wounding, which are known as DAMPs, can also elicit plant defense (Zipfel, 2014). Detection of different PAMPs/DAMPs by the corresponding PRRs at the plasma membrane activates signaling cascades, leading to transcriptional and physiological changes in host cells that prevent pathogen infection and establish PTI (Jones and Dangl, 2006; Macho and Zipfel, 2014; Bartels and Boller, 2015). In plants, PRRs are represented by RLKs and RLPs located at the cell surface, both of which require a co-receptor to form an active complex and initiate signaling (Machado et al., 2015). The best characterized co-receptors for PRR are members of LRR subfamily II of the RLK superfamily (LRRII-RLK subfamily). This family is represented by 13 members in the Arabidopsis genome, which can be divided into three closely related clusters: one representing five SOMATIC EMBRYOGENESIS RECEPTOR KINASES (SERK1-5), a cluster of LRR-RLKs of unknown function and a cluster of NUCLEAR-SHUTTLE PROTEIN-INTERACTING KINASES (NIK1-3; Zhang et al., 2006; Sakamoto et al., 2012). Among SERKs, SERK3, which is also termed BRASSINOSTEROID INSENSITIVE 1 (BRI1)-ASSOCIATED KINASE 1 (BAK1), is the most well-characterized subfamily member. SERK3 functions as a co-receptor of several PRRs, such as FLAGELLIN SENSING 2 (FLS2), ELONGATION FACTOR-thermo unstable (EF-Tu) receptor (EFR) or PEP1 receptor 1 (PEPR1), which perceive specific PAMPs/DAMPs and trigger or amplify bacterial/fungal PTI (Chinchilla et al., 2007; Heese et al., 2007; Roux et al., 2011; Wang et al., 2014). Upon PAMP perception, FLS2 and EFR form a ligand-induced complex with BAK1, which leads to rapid phosphorylation of both proteins (Chinchilla et al., 2007; Heese et al., 2007; Roux et al., 2011; Sun et al., 2013) and activation of immune responses, including production of ROS by the NADPH oxidase RBOHD, activation of the mitogen-activated protein kinase (MAPK) cascade, transcriptional reprogramming of defense genes and immunity to pathogens (Kadota et al., 2014; Li et al., 2014; Macho and Zipfel, 2014).

The mechanism of PTI in virus–host interactions is well characterized in animals. One of the best studied PRRs in mammals, Toll-like receptors (TLR), have important roles in antiviral defense via recognition of a different range of MAMPs, such viral RNA and DNA (Song and Lee, 2012). In contrast, the PTI pathway in plants remains unclear with regard to resistance against viruses, although studies describing an association of PTI in antiviral immunity have been recently reported (Yang et al., 2010; Kørner et al., 2013; Nicaise, 2014; Machado et al., 2015; Nicaise and Candresse, 2016; Niehl et al., 2016). Indeed, a complex set of typical PTI responses is induced in plants upon virus infection, including SA accumulation, ROS production, ion fluxes, defense gene (e.g., PR-1) activation, and callose deposition (for a review, see Nicaise, 2014). The PRR co-receptors BAK1

or BAK1-LIKE1 (BKK1) are required for antiviral immunity in Arabidopsis, and loss-of-function mutations in *BAK1* and *BKK1* result in enhanced susceptibility to TCV infection (Yang et al., 2010). Consistently, Arabidopsis *bak-1* mutants show increased susceptibility to three different RNA viruses, and crude extracts of virus-infected leaf tissues induce typical PTI responses in a BAK1-dependent manner (Kørner et al., 2013). The Arabidopsis double mutant *bak1-5 bkk1* displays increased viral accumulation when inoculated with *Plum pox virus* (PPV; Nicaise and Candresse, 2016). Furthermore, MAPK4, a negative regulator of plant PTI signaling, suppresses soybean defense against *Bean pod mottle virus* (BPMV; Liu et al., 2011), and chitosan, a deacetylated chitin derivative elicitor, is able to stimulate the plant immune response against viruses (Iriti and Varoti, 2014).

The current data suggest that viral components can act as PAMPs but do not eliminate the possibility that DAMPs produced in response to virus can potentially elicit PTI-based antiviral responses in plants. Recently, double-stranded RNA (dsRNA) and virus-derived dsRNA have been shown to function as viral PAMPs in Arabidopsis and to induce the PTI pathway (Niehl et al., 2016). Indeed, application of dsRNA to Arabidopsis leaf disks resulted in the induction of typical PTI responses, including MAPK activation, ethylene synthesis and defense gene expression. Furthermore, dsRNA treatment confers protection against viruses because plants inoculated with the synthetic dsRNA analog polyinosinic–polycytidylic acid, poly(I:C) together with *Oilseed rape mosaic virus* (ORMV) showed significantly reduced viral accumulation in treated leaves. Interestingly, dsRNA-mediated PTI appears to be independent of the RNA silencing pathway but does involve the co-receptor kinase SERK1. These findings relate membrane-associated signaling events with dsRNA-mediated PTI in plants (Niehl et al., 2016). Although plasma membrane-localized co-receptors of PRRs, such as BAK1, BKK1 and SERK1, have been shown to be involved in viral PTI, it remains to be determined how intracellular pathogens, which deliver PAMPs intracellularly, are perceived extracellularly.

The PTI pathway also contributes to antiviral immunity against PPV in Arabidopsis (Nicaise and Candresse, 2016). As a counteraction strategy, the CP from PPV appears to act as a PTI suppressor, impairing early immune responses such as the oxidative burst and enhanced expression of PTI-associated marker genes *in planta* during infection (Nicaise and Candresse, 2016). Therefore, PPV CP displays a virulence function that acts at the PTI level and antagonizes the Avr functions of many viral recognized by antiviral R proteins during elicitation of ETI (**Table 1**). These observations suggest that plant viruses also fit into the zigzag model of co-evolving pathogenic virulence strategies and plant defense responses that shape the two-branched innate immune system (Jones and Dangl, 2006).

Collectively, these data suggest the existence of PTI signaling mechanism targeting plant viruses and may represent a conserved process between plants and animals. Identification of PRR-mediated pathways as well as characterization of nucleic acid-sensing PRRs will shed light on the mechanism by which PTI is elicited in plants and its role in antiviral resistance.

TRANSMEMBRANE RECEPTOR-MEDIATED TRANSLATIONAL SUPPRESSION IN ANTIVIRAL IMMUNITY: UNIQUE AND SHARED PTI-LIKE FEATURES OF THE NIK1-MEDIATED ANTIVIRAL RESPONSE

The transmembrane receptor NIK was first identified as a virulence target of Nuclear Shuttle Protein (NSP) from *Begomovirus,* the largest genus of the *Geminiviridae* family (Fontes et al., 2004; Mariano et al., 2004). Similar to the PTI co-receptors BAK1 and SERK1, NIK receptors (NIK1, NIK2 and NIK3) belong to the LRRII-RLK subfamily and are involved in plant defenses against viruses (Fontes et al., 2004). Nevertheless, the mechanism by which NIK1 transduces the antiviral signal is completely different from the typical PTI signaling mediated by BAK1 or SERK1 and PRRs. Nonetheless, some similarities between these transduction pathways with regard to receptor activation, suppression and association with ETI have been observed (Machado et al., 2015, **Figure 2**).

NUCLEAR SHUTTLE PROTEIN-INTERACTING KINASE 1-mediated antiviral signaling is activated upon perception of begomovirus infection, which leads to phosphorylation of the NIK1 kinase at key threonine residues at positions 468 and 474 (Santos et al., 2009; Zorzatto et al., 2015). Thr-468 and Thr-474 are located within the conserved activation loop and align to the same positions as conserved BAK1 residues Thr-449 and Thr-455 and SERK1 residues Thr-462 and Thr-468, which are intramolecular targets for BAK1 and SERK1 kinase activation (Shah et al., 2001; Wang et al., 2005, 2008; Yun et al., 2009). Phosphorylation of the functional analogs NIK1 Thr-474, SERK1 Thr-468 and BAK1 Thr-455 is essential for receptor/co-receptor signaling, which may underscore a similar mechanism for activation (Shah et al., 2001; Wang et al., 2008; Santos et al., 2009; Yun et al., 2009; Brustolini et al., 2015). Nevertheless, unlike BAK1 or SERK1, phosphorylation at NIK1 Thr-474 leads to phosphorylation at Thr-469, which has an inhibitory effect, thereby providing a mechanism by which NIK1 modulates the extent of auto- and substrate phosphorylation. Although NIK1 is activated upon perception of virus infection, the molecular bases of such elicitation are unknown. Indeed, there is a complete lack of information with respect to the nature and identity of possible ligands or mechanisms that trigger or stabilize NIK1 dimerization or multimerization with receptors. Because viruses are intracellular pathogens and may not have access to the apoplast, it remains to be determined how the NIK1 extracellular domain, which is expected to drive ligand-dependent oligomerization of receptors and co-receptors, senses viruses intracellularly. Possible ligands that could perform this function are DAMPs, which would be secreted by plant cells upon virus perception. Alternatively, viral nucleic acid-derived PAMPs could intracellularly activate NIK1 kinase, a mechanism that would resemble virus-derived dsRNA-mediated activation of mammalian intracellular protein kinase R (PKR; Balachandran et al., 2000). Virus-derived nucleic acid PAMPs

could also activate NIK1-associated nucleic acid-sensing PRRs in endosomes derived from receptor internalization via endocytic pathways. In plant cells, the PRRs FLS2, ERR and PEPR have been shown to be internalized in a clathrin-dependent manner. Endocytosis requires the co-receptor BAK1 and depends on receptor activation (Mbengue et al., 2016). In a similar manner, the Avr factor Avr4 induces association of Cf-4 RLP with BAK1 to initiate receptor endocytosis and plant immunity (Postma et al., 2016).

In general, ligand-dependent phosphorylation and activation of RLKs require homo or heterodimerization of the receptors. In the case of BAK1 and SERK1, compelling evidence has revealed that they function primarily as co-receptors for receptor signaling not only in defense but also in development (Ma et al., 2016). As a member of the LRRII-RLK subfamily sharing conserved structural organization and biochemical activation properties with SERKs, NIK1 may also function as a co-receptor in immune active complexes. However, NIK1-containing antiviral signaling complexes have not been isolated, and a receptor partner for NIK1 has yet to be identified.

Begomovirus NSP binds *in vitro* and *in vivo* with the kinase domain of NIK1 to suppress NIK1 activity (Fontes et al., 2004; Brustolini et al., 2015). The NSP binding site corresponds to an 80-amino acid stretch (positions 422–502) of NIK1 that encompasses the putative Ser/Thr kinase active site (subdomain VIb–HrDvKssNxLLD) and the activation loop (subdomain VII–DFGAk/rx, plus subdomain VIII–GtxGyiaPEY; Fontes et al., 2004). Binding of NSP to the kinase domain promotes steric constraints that impair intermolecular phosphorylation at Thr-474 within the A-loop of NIK1, thereby suppressing its kinase activity. The NSP-mediated suppression of NIK1 kinase prevents activation of the NIK-mediated pathway and hence enhances the pathogenicity of begomoviruses in their hosts (Santos et al., 2009, 2010). In addition to acting as a virulence factor suppressing NIK1-mediated antiviral signaling, NSP from the begomovirus *Bean dwarf mosaic virus* (BDMV) has been demonstrated to function as an Avr gene and elicit HR in *Phaseolus vulgaris* (Garrido-Ramirez and Gilbertson, 1998). According to the zigzag evolutionary model of plant innate immunity (Jones and Dangl, 2006), the involvement and activation of ETI in plant-virus interactions (NSP in resistant bean genotypes) is conceptually associated with successful PTI inhibition (NIK1 signaling) by a viral effector (NSP). This interpretation further substantiates the notion that NIK1-mediated antiviral signaling shows features of PTI-like mechanisms.

Despite similarities in the activation and suppression mechanisms of PTI and NIK1-mediated antiviral signaling, the downstream events of NIK1 activation are quite distinct from the typical PTI response. In fact, activation of NIK1 signaling by constitutive or inducible expression of the gain-of-function T474D NIK1 mutant, which is not inhibited by viral NSP, results in a massive down-regulation of translation machinery-related genes, suppression of host global translation and enhanced broad-spectrum tolerance to begomoviruses in Arabidopsis and tomato (Brustolini et al., 2015; Zorzatto et al., 2015). T474D-mediated suppression of global translation is associated with a decrease in host and viral mRNA in actively translating polysomes. Therefore, begomovirus is not capable of sustaining high levels of viral mRNA translation in T474D-expressing lines, indicating that suppression of global protein synthesis may effectively protect plant cells against DNA viruses.

Progress toward deciphering the mechanism of the translational control branch of NIK1 signaling includes identification of the downstream effectors, RPL10 and LIMYB (Rocha et al., 2008; Zorzatto et al., 2015). RPL10 was isolated based on its capacity to interact with NIK1 and was genetically and biochemically linked to the NIK1-mediated signaling pathway (Carvalho et al., 2008; Rocha et al., 2008). Consistent with a role for RPL10 in antiviral defense, loss of *RPL10* function recapitulates the *nik1* enhanced susceptibility phenotype to begomovirus infection, as *rpl10* knockout lines develop severe symptoms similar to those of *nik1* and display a similar infection rate (Carvalho et al., 2008; Rocha et al., 2008). NIK1 activation mediates RPL10 phosphorylation and consequent translocation of the RP from the cytoplasm to the nucleus. The regulated nucleocytoplasmic shuttling of RPL10 depends on NIK1 kinase activity and on the phosphorylation status of the RP (Carvalho et al., 2008). Mutations that impact NIK1 activity similarly affect the capacity of NIK1 to mediate translocation of RPL10 to the nucleus and to transduce an antiviral signal. In the nucleus, RPL10 interacts with LIMYB to form a transcription-repressing complex that specifically down-regulates expression of translational machinery-related genes, such as RP genes. This down-regulation of RP genes results in global suppression of host translation and enhanced tolerance to begomoviruses. Expression of the gain-of-function T474D mutant also results in repression of the same set of LIMYB-regulated RP genes, but T474D requires the function of LIMYB for RP repression. In addition, loss of LIMYB function releases the repression of translation-related genes and increases susceptibility to *Cabbage leaf curl virus* (CaLCuV) infection (Zorzatto et al., 2015). Collectively, these results provide both genetic and biochemical evidence that LIMYB functions as a downstream component of the NIK1-mediated signaling pathway linking NIK1 activation to global translation suppression and tolerance to begomoviruses.

Although NIK1 is structurally similar to SERKs, the mechanism of NIK1-mediated antiviral defense is distinct from that of BAK1-mediated PTI. The current model for NIK1-mediated antiviral signaling states that, in response to virus infection, NIK1 undergoes homo- or heterodimerization to promote phosphorylation of the activation loop (**Figure 2**). Activated NIK1 mediates phosphorylation and consequent translocation of RPL10 to the nucleus, where it interacts with LIMYB to fully repress RP gene expression. Prolonged down-regulation of RP gene expression leads to suppression of global host translation. DNA viruses, such as begomoviruses, cannot escape this translational regulatory mechanism of plant cells, and viral mRNAs are not translated efficiently, thereby compromising infection. NSP acts as a virulence factor and suppresses the kinase activity of NIK1 to overcome the NIK1-mediated immune response. NSP from the begomovirus BDMV has also been shown to function as an Avr factor that activates typical ETI responses in resistant bean genotypes. Therefore, NSP may link the suppression of NIK1 signaling with activation of ETI

responses in accordance with the zigzag evolutionary model of plant innate immunity, although the NIK1-mediated antiviral signaling may represent a new evolved branch of plant antiviral immunity, which relies on suppression of translation for defense.

CONCLUSION

Innate immunity against plant viruses and its underlying mechanisms have attracted the attention of breeders and scientists. Accordingly, there is a growing list of R genes against viruses, and our knowledge regarding the mechanisms of R gene-mediated defenses has advanced considerably over the last decade. However, in comparison with R genes against non-viral pathogens, the number of well-studied examples of antiviral R genes is still limited with respect to an understanding of the level of specialization of dominant resistance against viruses and the boundaries of features shared with non-viral ETI. Even more limited is our understanding of viral PTI in plants. Recent studies have provided insights into plant viral PTI. For example, it is now known that several components of bacterial and fungal PTI participate also in viral PTI. These include the co-receptor SERKs, BAK1 and SERK1, and the MAPK4 negative regulator, in addition to common effects of non-viral PTI that are also elicited during virus infection. Nevertheless, our knowledge about the dynamics between the virulence strategy of viruses and the plant immune system is still rudimentary, and several steps in the mechanism of antiviral innate immunity are still unknown. Indeed, although non-self RNA motifs appear to function as PAMPs from RNA viruses, we do not know the identities of virus-derived PAMPs or plant-derived DAMPs that would induce antiviral PTI. The repertoire of viral suppressors of PTI is limited to the CP from PPV and perhaps to NSP from begomoviruses. Furthermore, antiviral PRRs have not been identified, and mechanisms by which intracellular pathogens that have no access to the apoplast are sensed extracellularly are unknown. A better understanding of the repertoire of virus effectors (Avr factor) and NB-LRR host targets (R proteins) and their mode of action in activating ETI and/or suppressing PTI will help to define the evolutionary pressure acting upon the host and viruses and to determine how to deploy the immune system for more efficient control of virus infection. We also need to define NIK1-mediated suppression of translation as a general or virus-specific antiviral strategy in plants. To date, a sustained NIK1 pathway has been shown to be effective against begomoviruses, one of the largest groups of plant DNA viruses, which cannot circumvent the regulatory mechanism of host translation. In this regard, the intrinsic capacity of agronomically relevant crops to withstand the deleterious effects of suppression of global translation must be considered as a relevant agronomic trait if we are to use the translational control branch of NIK1-mediated antiviral signaling for crop protection against begomoviruses.

AUTHOR CONTRIBUTIONS

BG and IC wrote the first draft of the manuscript; JM wrote the ETI section; AS edited the manuscript and EF edited the manuscript.

ACKNOWLEDGMENTS

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico [573600/2008-2 and 447578/2014-6 to E.P.B.F.) and the Fundação de Amparo à Pesquisa do Estado de Minas Gerais [CBB-APQ-03175-13 and CBB-APQ-01491-14 to EF]. BG and IC were supported by CNPq graduate fellowships, and JM is a recipient of a CAPES postdoctoral fellowship.

REFERENCES

Aarts, N., Metz, M., Holub, E., Staskawicz, B. J., Daniels, M. J., and Parker, J. E. (1998). Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10306–10311. doi: 10.1073/pnas.95.17.10306

Anagnostou, K., Jahn, M., and Perl-Treves, R. (2000). Inheritance and linkage analysis of resistance to Zucchini yellow mosaic virus, *Watermelon mosaic virus*, *Papaya ringspot virus* and powdery mildew in melon. *Euphytica* 116, 265–270. doi: 10.1023/A:1004005716806

Balachandran, S., Roberts, P. C., Brown, L. E., Truong, H., Pattnaik, A. K., Archer, D. R., et al. (2000). Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. *Immunity* 13, 129–141. doi: 10.1016/S1074-7613(00)00014-5

Bartels, S., and Boller, T. (2015). Quo vadis, Pep? Plant elicitor peptides at the crossroads of immunity, stress, and development. *J. Exp. Bot.* 66, 5183–5193. doi: 10.1093/jxb/erv180

Bendahmane, A., Kanyuka, K., and Baulcombe, D. C. (1999). The Rx gene from potato controls separate virus resistance and cell death responses. *Plant Cell* 11, 781–791. doi: 10.1105/tpc.11.5.781

Bendahmane, A., Köhm, B. A., Dedi, C., and Baulcombe, D. C. (1995). The coat protein of potato virus X is a strain-specific elicitor of Rx1-mediated virus resistance in potato. *Plant J.* 8, 933–941. doi: 10.1046/j.1365-313X.1995.8060933.x

Bendahmane, A., Querci, M., Kanyuka, K., and Baulcombe, D. C. (2000). *Agrobacterium* transient expression system as a tool for the isolation of disease resistance genes: application to the Rx2 locus in potato. *Plant J.* 21, 73–81. doi: 10.1046/j.1365-313x.2000.00654.x

Bent, A. F., and Mackey, D. (2007). Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. *Annu. Rev. Phytopathol.* 45, 399–436. doi: 10.1146/annurev.phyto.45.062806.094427

Bigeard, J., Colcombet, J., and Hirt, H. (2015). Signaling mechanisms in pattern-triggered immunity (PTI). *Mol. Plant.* 8, 521–539. doi: 10.1016/j.molp.2014.12.022

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60, 379–406. doi: 10.1146/annurev.arplant.57.032905.105346

Bonardi, V., Cherkis, K., Nishimura, M. T., and Dangl, J. L. (2012). A new eye on NLR proteins: focused on clarity or diffused by complexity? *Curr. Opin. Immunol.* 24, 41–50. doi: 10.1016/j.coi.2011.12.006

Botër, M., Amigues, B., Peart, J., Breuer, C., Kadota, Y., Casais, C., et al. (2007). Structural and functional analysis of SGT1 reveals that its interaction with HSP90 is required for the accumulation of Rx, an R protein

involved in plant immunity. *Plant Cell* 19, 3791–3804. doi: 10.1105/tpc.107.050427

Brommonschenkel, S. H., Frary, A., and Tanksley, S. D. (2000). The broad-spectrum *Tospovirus* resistance gene Sw-5 of tomato is a homolog of the root-knot nematode resistance gene Mi. *Mol. Plant Microbe Interact.* 13, 1130–1138. doi: 10.1094/MPMI.2000.13.10.1130

Brotman, Y., Normantovich, M., Goldenberg, Z., Zvirin, Z., Kovalski, I., Stovbun, N., et al. (2013). Dual resistance of melon to *Fusarium oxysporum* races 0 and 2 and to *Papaya ringspot virus* is controlled by a pair of head-to-head- oriented NB-LRR genes of unusual architecture. *Mol. Plant* 6, 235–238. doi: 10.1093/mp/sss121

Brustolini, O. J. B., Machado, J. P. B., Condori-Apfata, J. A., Coco, D., Deguchi, M., Loriato, V. A., et al. (2015). Sustained NIK-mediated antiviral signalling confers broad-spectrum tolerance to begomoviruses in cultivated plants. *Plant Biotechnol. J.* 13, 1300–1311. doi: 10.1111/pbi.12349

Burch-Smith, T. M., Schiff, M., Caplan, J. L., Tsao, J., Czymmek, K., and Dinesh-Kumar, S. P. (2007). A novel role for the TIR domain in association with pathogen-derived elicitors. *PLoS Biol.* 5:e68. doi: 10.1371/journal.pbio.0050068

Butterbach, P., Verlaan, M. G., Dullemans, A., Lohuis, D., Visser, R. G. F., Bai, Y., et al. (2014). *Tomato yellow leaf curl virus* resistance by Ty-1 involves increased cytosine methylation of viral genomes and is compromised by *Cucumber mosaic virus* infection. *Proc. Natl. Acad. Sci. U.S.A.* 111, 12942–12947. doi: 10.1073/pnas.1400894111

Candresse, T., Marais, A., Faure, C., Dubrana, M. P., Gombert, J., and Bendahmane, A. (2010). multiple coat protein mutations abolish recognition of Pepino mosaic potexvirus (PepMV) by the potato rx resistance gene in transgenic tomatoes. *Mol. Plant Microbe Interact.* 23, 376–383. doi: 10.1094/MPMI-23-4-0376

Caplan, J. L., Mamillapalli, P., Burch-Smith, T. M., Czymmek, K., and Dinesh-Kumar, S. P. (2008). Chloroplastic protein NRIP1 mediates innate immune receptor recognition of a viral effector. *Cell* 132, 449–462. doi: 10.1016/j.cell.2007.12.031

Carr, J. P., Lewsey, M. G., and Palukaitis, P. (2010). Chapter3 – signaling in induced resistance. *Adv. Virus Res.* 76, 57–121. doi: 10.1016/S0065-3527(10)76003-6

Carvalho, C. M., Santos, A. A., Pires, S. R., Rocha, S. R., Saraiva, D. I., Machado, J. P., et al. (2008). Regulated nuclear trafficking of rpL10A mediated by NIK1 represents a defense strategy of plant cells against viruses. *PLoS Pathog.* 4:e1000247. doi: 10.1371/journal.ppat.1000247

Chandra-Shekara, A. C., Navarre, D., Kachroo, A., Kang, H.-G., Klessig, D., and Kachroo, P. (2004). Signaling requirements and role of salicylic acid in HRT- and rrt-mediated resistance to *Turnip crinkle virus* in *Arabidopsis*. *Plant J.* 40, 647–659. doi: 10.1111/j.1365-313X.2004.02241.x

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J. D., et al. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448, 497–500. doi: 10.1038/nature05999

Chisholm, S. T., Mahajan, S. K., Whitham, S. A., Yamamoto, M. L., and Carrington, J. C. (2000). Cloning of the *Arabidopsis* RTM1 gene, which controls restriction of long-distance movement of *Tobacco etch virus*. *Proc. Natl. Acad. Sci. U.S.A.* 97, 489–494. doi: 10.1073/pnas.97.1.489

Chu, M., Desvoyes, B., Turina, M., Noad, R., and Scholthof, H. B. (2000). Genetic dissection of *Tomato bushy stunt virus* p19-protein-mediated host-dependent symptom induction and systemic invasion. *Virology* 266, 79–87. doi: 10.1006/viro.1999.0071

Cole, A. B., Király, L., Ross, K., and Schoelz, J. E. (2001). Uncoupling resistance from cell death in the hypersensitive response of *Nicotiana* species to cauliflower mosaic virus infection. *Mol. Plant Microbe Interact.* 14, 31–41. doi: 10.1094/MPMI.2001.14.1.31

Coll, N. S., Epple, P., and Dangl, J. L. (2011). Programmed cell death in the plant immune system. *Cell Death Differ.* 18, 1247–1256. doi: 10.1038/cdd.2011.37

Cooley, M. B., Pathirana, S., Wu, H. J., Kachroo, P., and Klessig, D. F. (2000). Members of the *Arabidopsis* HRT/RPP8 family of resistance genes confer resistance to both viral and oomycete pathogens. *Plant Cell* 12, 663–676. doi: 10.1105/tpc.12.5.663

Culver, J. N., and Padmanabhan, M. S. (2007). Virus-induced disease: altering host physiology one interaction at a time. *Annu. Rev. Phytopathol.* 45, 221–243. doi: 10.1146/annurev.phyto.45.062806.094422

Dangl, J. L., Horvath, D. M., and Staskawicz, B. J. (2013). Pivoting the plant immune system from dissection to deployment. *Science* 341, 746–751. doi: 10.1126/science.1236011

Dangl, J. L., and Jones, J. D. (2001). Plant pathogens and integrated defence responses to infection. *Nature* 411, 826–833. doi: 10.1038/35081161

de Ronde, D., Butterbac, P., and Kormelink, R. (2014). Dominant resistance against plant viruses. *Front. Plant Sci.* 5:307. doi: 10.3389/fpls.2014.00307

Decroocq, V., Salvador, B., Sicard, O., Glasa, M., Cosson, P., Svanella-Dumas, L., et al. (2009). The determinant of potyvirus ability to overcome the RTM resistance of *Arabidopsis thaliana* maps to the N-terminal region of the coat protein. *Mol. Plant Microbe Interact.* 22, 1302–1311. doi: 10.1094/MPMI-22-10-1302

Dodds, P. N., and Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant–pathogen interactions. *Nat. Rev. Genet.* 11, 539–548. doi: 10.1038/nrg2812

Erickson, F. L., Holzberg, S., Calderon-Urrea, A., Handley, V., Axtell, M., Corr, C., et al. (1999). The helicase domain of the TMV replicase proteins induces the N-mediated defense response in tobacco. *Plant J.* 18, 67–75. doi: 10.1046/j.1365-313X.1999.00426.x

Falk, A., Feys, B. J., Frost, L. N., Jones, J. D. G., Daniels, M. J., and Parker, J. E. (1999). EDS1, an essential component of R gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. *Proc. Natl. Acad. Sci. U.S.A.* 96, 3292–3297. doi: 10.1073/pnas.96.6.3292

Farnham, G., and Baulcombe, D. C. (2006). Artificial evolution extends the spectrum of viruses that are targeted by a disease-resistance gene from potato. *Proc. Natl. Acad. Sci. U.S.A.* 103, 18828–18833. doi: 10.1073/pnas.0605777103

Feys, B. J., Moisan, L. J., Newman, M. A., and Parker, J. E. (2001). Direct interaction between the *Arabidopsis* disease resistance signaling proteins, EDS1 and PAD4. *EMBO J.* 20, 5400–5411. doi: 10.1093/emboj/20.19.5400

Feys, B. J., Wiermer, M., Bhat, R. A., Moisan, L. J., Medina-Escobar, N., Neu, C., et al. (2005). *Arabidopsis* SENESCENCE- ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. *Plant Cell* 17, 2601–2613. doi: 10.1105/tpc.105.033910

Flor, H. (1971). Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* 9, 275–296. doi: 10.1146/annurev.py.09.090171.001423

Fontes, E. P. B., Santos, A. A., Luz, D. F., Waclawovsky, A. J., and Chory, J. (2004). The geminivirus nuclear shuttle protein is a virulence factor that suppresses transmembrane receptor kinase activity. *Genes Dev.* 18, 2545–2556. doi: 10.1101/gad.1245904

Garrido-Ramirez, E. R., and Gilbertson, R. L. (1998). First report of tomato mottle geminivirus infecting tomato in Yucatan, Mexico. *Plant Dis.* 82:592. doi: 10.1094/PDIS.1998.82.5.592B

Goelet, P., Lomonossoff, G. P., Butler, P. J., Akam, M. E., Gait, M. J., and Karn, J. (1982). Nucleotide sequence of tobacco mosaic virus RNA. *Proc. Natl. Acad. Sci. U.S.A.* 79, 5818–5822. doi: 10.1073/pnas.79.19.5818

Hallwass, M., de Oliveira, A. S., de Campos Dianese, E., Lohuis, D., Boiteux, L. S., Inoue-Nagata, A. K., et al. (2014). The *Tomato spotted wilt virus* cell-to-cell movement protein (NS) triggers a hypersensitive response in Sw-5-containing resistant tomato lines and in *Nicotiana benthamiana* transformed with the functional Sw-5b resistance gene copy. *Mol. Plant Pathol.* 15, 871–880. doi: 10.1111/mpp.12144

Hao, W., Collier, S. M., Moffett, P., and Chai, J. (2013). Structural basis for the interaction between the potato virus X resistance protein (Rx) and its cofactor Ran GTPase-activating protein 2 (RanGAP2). *J. Biol. Chem.* 288, 35868–35876. doi: 10.1074/jbc.M113.517417

Hayes, A. J., Jeong, S. C., Gore, M. A., Yu, Y. G., Buss, G. R., Tolin, S. A., et al. (2004). Recombination within a nucleotide-binding-site/leucine-rich-repeat gene cluster produces new variants conditioning resistance to *Soybean mosaic virus* in soybeans. *Genetics* 166, 493–503. doi: 10.1534/genetics.166.1.493

Heese, A., Hann, D. R., Gimenez-Ibanez, S., Jones, A. M., He, K., Li, J., et al. (2007). The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proc. Natl. Acad. Sci. U.S.A.* 104, 12217–12222. doi: 10.1073/pnas.0705306104

Hogenhout, S. A., van der Hoorn, R. A. L., Terauchi, R., and Kamoun, S. (2009). Emerging concepts in effector biology of plant-associated organisms. *Mol. Plant Microbe Interact.* 22, 115–122. doi: 10.1094/mpmi-22-2-0115

Holmes, F. O. (1929). Local lesions in tobacco mosaic. *Bot. Gaz.* 87, 39–55. doi: 10.1086/333923

Holmes, F. O. (1938). Inheritance of resistance to tobacco-mosaic disease in tobacco. *Phytopathology* 28, 553–561.

Hoser, R., Lichocka, M., Zurczak, M., Hennig, J., and Krzymowska, M. (2014). Emerging role of SGT1 as a regulator of NB-LRR-receptor nucleocytoplasmic partitioning. *Plant Signal. Behav.* 9:e28724. doi: 10.4161/psb.28724

Hoser, R., Zurczak, M., Lichocka, M., Zuzga, S., Dadlez, M., Samuel, M. A., et al. (2013). Nucleocytoplasmic partitioning of tobacco N receptor is modulated by SGT1. *New Phytol.* 200, 158–171. doi: 10.1111/nph.12347

Howden, A. J., and Huitema, E. (2012). Effector-triggered post-translational modifications and their role in suppression of plant immunity. *Front. Plant Sci.* 3:160. doi: 10.3389/fpls.2012.00160

Huisman, M. J., Linthorst, H. J., Bol, J. F., and Cornelissen, J. C. (1988). The complete nucleotide sequence of potato virus X and its homologies at the amino acid level with various plus-stranded RNA viruses. *J. Gen. Virol.* 69, 1789–1798. doi: 10.1099/0022-1317-69-8-1789

Iriti, M., and Varoti, E. M. (2014). Chitosan-induced antiviral activity and innate immunity in plants. *Environ. Sci. Pollut. Res. Int.* 22, 2935–2944. doi: 10.1007/s11356-014-3571-7

Ishibashi, K., and Ishikawa, M. (2013). The resistance protein Tm-1 inhibits formation of a *Tomato mosaic virus* replication protein-host membrane protein complex. *J. Virol.* 87, 7933–7939. doi: 10.1128/JVI.00743-13

Ishibashi, K., and Ishikawa, M. (2014). Mechanisms of *Tomato mosaic virus* RNA replication and its inhibition by the host resistance factor Tm-1. *Curr. Opin. Virol.* 9, 8–13. doi: 10.1016/j.coviro.2014.08.005

Ishibashi, K., Kezuka, Y., Kobayashi, C., Kato, M., Inoue, T., Nonaka, T., et al. (2014). Structural basis for the recognition-evasion arms race between *Tomato mosaic virus* and the resistance gene Tm-1. *Proc. Natl. Acad. Sci. U.S.A.* 111, e3486–e3495. doi: 10.1073/pnas.1407888111

Ishibashi, K., Masuda, K., Naito, S., Meshi, T., and Ishikawa, M. (2007). An inhibitor of viral RNA replication is encoded by a plant resistance gene. *Proc. Natl. Acad. Sci. U.S.A.* 104, 13833–13838. doi: 10.1073/pnas.0703203104

Ishibashi, K., Mawatari, N., Miyashita, S., Kishino, H., Meshi, T., and Ishikawa, M. (2012). Coevolution and hierarchical interactions of *Tomato mosaic virus* and the resistance gene Tm-1. *PLoS Pathog.* 8:e1002975. doi: 10.1371/journal.ppat.1002975

Ishibashi, K., Naitob, S., Meshia, T., and Ishikawa, M. (2009). An inhibitory interaction between viral and cellular proteins underlies the resistance of tomato to non adapted tobamoviruses. *Proc. Natl. Acad. Sci. U.S.A.* 106, 8778–8783. doi: 10.1073/pnas.0809105106

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Kadota, Y., and Shirasu, K. (2012). The HSP90 complex of plants. *Biochim. Biophys. Acta* 1823, 689–697. doi: 10.1016/j.bbamcr.2011.09.016

Kadota, Y., Sklenar, J., Derbyshire, P., Stransfeld, L., Asai, S., Ntoukakis, V., et al. (2014). Direct Regulation of the NADPH Oxidase RBOHD by the PRR-Associated Kinase BIK1 during Plant Immunity. *Mol. Cell* 54, 43–55. doi: 10.1016/j.molcel.2014.02.021

Kato, M., Ishibashi, K., Kobayashi, C., Ishikawa, M., and Katoh, E. (2013). Expression, purification, and functional characterization of an N-terminal fragment of the *Tomato mosaic virus* resistance protein Tm-1. *Protein Expr. Purif.* 89, 1–6. doi: 10.1016/j.pep.2013.02.001

Kim, S.-B., Kang, W. H., Huy, H. N., Yeom, S. I., An, J. T., Kim, S., et al. (2016). Divergent evolution of multiple virus-resistance genes from a progenitor in *Capsicum* spp. *New Phytol.* doi: 10.1111/nph.14177 [Epub ahead of print].

Kim, S.-B., Lee, H.-Y., Seo, S., Lee, J. H., and Choi, D. (2015). RNA-dependent RNA polymerase (NIb) of the potyviruses is an avirulence factor for the broad-spectrum resistance gene Pvr4 in *Capsicum annuum* cv. CM334. *PLoS ONE* 10:e0119639. doi: 10.1371/journal.pone.0119639

Kørner, C. J., Klauser, D., Niehl, A., Dominguez-Ferreras, A., Chinchilla, D., Boller, T., et al. (2013). The immunity regulator BAK1 contributes to resistance against diverse RNA viruses. *Mol. Plant Microbe Interact.* 26, 1271–1280. doi: 10.1094/MPMI-06-13-0179-R

Lanfermeijer, F. C., Dijkhuis, J., Sturre, M. J., de Haan, P., and Hille, J. (2003). Cloning and characterization of the durable *Tomato mosaic virus* resistance gene Tm-222 from *Lycopersicon esculentum*. *Plant Mol. Biol.* 52, 1037–1049. doi: 10.1023/A:1025434519282

Li, L., Li, M., Liping, Y., Zhou, Z., Liang, X., Liu, Z., et al. (2014). The FLS2-associated kinase BIK1 directly phosphorylates the NADPH oxidase RbohD to control plant immunity. *Cell Host Microbe* 15, 329–338. doi: 10.1016/j.chom.2014.02.009

Liu, J. Z., Horstman, H. D., Braun, E., Graham, M. A., Zhang, C., Navarre, D., et al. (2011). Soybean homologs of MPK4 negatively regulate defense responses and positively regulate growth and development. *Plant Physiol.* 157, 1363–1378. doi: 10.1104/pp.111.185686

Liu, Y., Burch-Smith, T., Schiff, M., Feng, S., and Dinesh-Kumar, S. P. (2004). Molecular chaperone Hsp90 associates with resistance protein N and its signaling proteins SGT1 and Rar1 to modulate an innate immune response in plants. *J. Biol. Chem.* 279, 2101–2108. doi: 10.1074/jbc.M310029200

Ma, J. F., Hou, X. L., Xiao, D., Qi, L., Wang, F., Sun, F. F., et al. (2010). Cloning and characterization of the BcTuR3 gene related to resistance to *Turnip mosaic virus* (TuMV) from non-heading chinese cabbage. *Plant Mol. Biol. Rep.* 28, 588–596. doi: 10.1007/s11105-010-0183-3

Ma, X., Xu, G., He, P., and Shan, L. (2016). SERKing coreceptors for receptors. *Plant Sci.* 21, 1017–1033. doi: 10.1016/j.tplants.2016.08.014

Machado, J. P. B., Brustolini, O. J. B., Mendes, G. C., Santos, A. A., and Fontes, E. P. B. (2015). NIK1, a host factor specialized in antiviral defense or a novel general regulator of plant immunity? *Bioessays* 37, 1236–1242. doi: 10.1002/bies.201500066

Macho, A. P., and Zipfel, C. (2014). Plant PRRs and the activation of innate immune signaling. *Mol. Cell* 54, 263–272. doi: 10.1016/j.molcel.2014.03.028

Maiti, S., Paul, S., and Pal, A. (2012). Isolation, characterization, and structure anal- ysis of a non-TIR-NBS-LRR encoding candidate gene from MYMIV-resistant *Vigna* mungo. *Mol. Biotechnol.* 52, 217–233. doi: 10.1007/s12033-011-9488-1

Mandadi, K. K., and Scholthof, K.-B. G. (2012). Characterization of a viral synergism in the monocot *Brachypodium* reveals distinctly altered host molecular processes associated with disease. *Plant Physiol.* 160, 1432–1452. doi: 10.1104/pp.112.204362

Mandadi, K. K., and Scholthof, K. B. G. (2013). Plant immune responses against viruses: How does a virus cause disease? *Plant Cell* 25, 1489–1505. doi: 10.1105/tpc.113.111658

Margaria, P., Ciuffo, M., Pacifico, D., and Turina, M. (2007). Evidence that the nonstructural protein of *Tomato spotted wilt virus* is the avirulence determinant in the interaction with resistant pepper carrying the Tsw gene. *Mol. Plant Microbe Interact.* 20, 547–558. doi: 10.1094/MPMI-20-5-0547

Mariano, A. C., Andrade, M. O., Santos, A. A., Carolino, S. M. B., Oliveira, M. L., Baracat-Pereira, M. C., et al. (2004). Identification of a novel receptor-like protein kinase that interacts with a geminivirus nuclear shuttle protein. *Virology* 318, 24–31. doi: 10.1016/j.virol.2003.09.038

Mbengue, M., Bourdais, G., Gervasi, F., Beck, M., Zhou, J., Spallek, T., et al. (2016). Clathrin-dependent endocytosis is required for immunity mediated by pattern recognition receptor kinases. *Proc. Natl. Acad. Sci. U.S.A.* 113, 11034–11039. doi: 10.1073/pnas.1606004113

Mestre, P., and Baulcombe, D. C. (2006). Elicitor-mediated oligomerization of the tobacco N disease resistance protein. *Plant Cell* 18, 491–501. doi: 10.1105/tpc.105.037234

Mur, L. A. J., Kenton, P., Lloyd, A. J., Ougham, H., and Prats, E. (2008). The hypersensitive response; the centenary is upon us but how much do we know? *J. Exp. Bot.* 59, 501–520. doi: 10.1093/jxb/erm239

Nicaise, V. (2014). Crop immunity against viruses: outcomes and future challenges. *Front. Plant Sci.* 5:660. doi: 10.3389/fpls.2014.00660

Nicaise, V., and Candresse, T. (2016). *Plum pox virus* capsid protein suppresses plant pathogen-associated molecular pattern (PAMP)-triggered immunity. *Mol. Plant Pathol.* doi: 10.1111/mpp.12447 [Epub ahead of print].

Niehl, A., Wyrsch, I., Boller, T., and Heinlein, M. (2016). Double-stranded RNAs induce a pattern-triggered immune signaling pathway in plants. *New Phytol.* 211, 1008–1019. doi: 10.1111/nph.13944

Osman, T. A., and Buck, K. W. (1996). Complete replication in vitro of tobacco mosaic virus RNA by a template-dependent, membrane-bound RNA polymerase. *J. Virol.* 70, 6227–6234.

Oßwald, W., Fleischmann, F., Rigling, D., Coelho, A. C., Cravador, A., Diez, J., et al. (2014). Strategies of attack and defence in woody plant–Phytophthora interactions. *For. Pathol.* 44, 169–190. doi: 10.1111/efp.12096

Padgett, H. S., Watanabe, Y., and Beachy, R. N. (1997). Identification of the TMV replicase sequence that activates the N gene-mediated hypersensitive response. *Mol. Plant Microbe Interact.* 10, 709–715. doi: 10.1094/MPMI.1997.10.6.709

Padmanabhan, M. S., Ma, S., Burch-Smith, T. M., Czymmek, K., Huijser, P., and Dinesh-Kumar, S. P. (2013). Novel positive regulatory role for the SPL6 transcription factor in the N TIR-NB-LRR receptor-mediated plant innate immunity. *PLoS Pathog.* 9:e1003235. doi: 10.1371/journal.ppat.1003235

Pallas, V., and García, J. A. (2011). How do plant viruses induce disease? Interactions and interference with host components. *J. Gen. Virol.* 92, 2691–2705. doi: 10.1099/vir.0.034603-0

Peiro, A., Canizares, M. C., Rubio, L., Lopez, C., Moriones, E., Aramburu, J., et al. (2014). The movement protein (NSm) of *Tomato spotted wilt virus* is the avirulence determinant in the tomato Sw-5 gene-based resistance. *Mol. Plant Pathol.* 15, 802–813. doi: 10.1111/mpp.12142

Postma, J., Liebrand, T. W., Bi, G., Evrard, A., Bye, R. R., Mbengue, M., et al. (2016). Avr4 promotes Cf-4 receptor-like protein association with the BAK1/SERK3 receptor-like kinase to initiate receptor endocytosis and plant immunity. *New Phytol.* 210, 627–642. doi: 10.1111/nph.13802

Rairdan, G. J., Collier, S. M., Sacco, M. A., Baldwin, T. T., Boettrich, T., and Moffett, P. (2008). The coiled-coil and nucleotide binding domains of the potato rx disease resistance protein function in pathogen recognition and signaling. *The Plant Cell* 20, 739–751. doi: 10.1105/tpc.107.056036

Reimer-Michalski, E. M., and Conrath, U. (2016). Innate immune memory in plants. *Semin. Immunol.* 4, 319–327. doi: 10.1016/j.smim.2016.05.006

Ren, T., Qu, F., and Morris, T. J. (2000). HRT gene function requires interaction between a NAC protein and viral capsid protein to confer resistance to *Turnip crinkle virus*. *Plant Cell* 12, 1917–1926. doi: 10.1105/tpc.12.10.1917

Rocha, C. S., Santos, A. A., Machado, J. P. B., and Fontes, E. P. B. (2008). The ribosomal protein L10/QM-like protein is a component of the NIK-mediated antiviral signaling. *Virology* 380, 165–169. doi: 10.1016/j.virol.2008.08.005

Ronde, D., Butterbach, P., Lohuis, D., Hedil, M., van Lent, J. W., and Kormelink, R. (2013). Tsw gene- based resistance is triggered by a functional RNA silencing suppressor protein of the *Tomato spotted wilt virus*. *Mol Plant Pathol.* 14, 405–415. doi: 10.1111/mpp.12016

Ronde, D., Pasquier, A., Ying, S., Butterbach, P., Lohuis, D., and Kormelink, R. (2014). Analysis of *Tomato spotted wilt virus* NSs protein indicates the importance of the N-terminal domain for avirulence and RNA silencing suppression. *Mol. Plant Pathol.* 15, 185–195. doi: 10.1111/mpp.12082

Ross, A. F. (1961). Systemic acquired resistance induced by localized virus infections in plants. *Virology* 14, 340–358. doi: 10.1016/0042-6822(61)90318-X

Roux, F., Voisin, D., Badet, T., Balagué, C., Barlet, X., Huard-Chauveau, C., et al. (2014). Resistance to phytopathogens e tutti quanti: placing plant quantitative disease resistance on the map. *Mol. Plant Pathol.* 15, 427–432. doi: 10.1111/mpp.12138

Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N., et al. (2011). The *Arabidopsis* leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. *Plant Cell* 23, 2440–2455. doi: 10.1105/tpc.111.084301

Sakamoto, T., Deguchi, M., Brustolini, O. J. B., Santos, A. A., Silva, F. F., and Fontes, E. P. (2012). The tomato RLK superfamily: phylogeny and functional predictions about the role of the LRRII-RLK subfamily in antiviral defense. *BMC Plant Biol.* 12:229. doi: 10.1186/1471-2229-12-229

Santos, A. A., Carvalho, C. M., Florentino, L. H., Ramos, H. J. O., and Fontes, E. P. (2009). Conserved threonine residues within the A-loop of the receptor NIK differentially regulate the kinase function required for antiviral signaling. *PLoS ONE* 4:e5781. doi: 10.1371/journal.pone.0005781

Santos, A. A., Lopes, K. V. G., Apfata, J. A. C., and Fontes, E. P. B. (2010). NSP-interacting kinase, NIK: a transducer of plant defence signalling. *J. Exp. Bot.* 61, 3839–3845. doi: 10.1093/jxb/erq219

Schwessinger, B., and Ronald, P. C. (2012). Plant Innate Immunity: perception of Conserved Microbial Signatures. *Annu. Rev. Plant Biol.* 63, 451–482. doi: 10.1146/annurev-arplant-042811-105518

Shah, K., Vervoort, J., and de Vries, S. C. (2001). Role of threonines in the *Arabidopsis thaliana* somatic embryogenesis receptor kinase 1 activation loop in phosphorylation. *J. Biol. Chem.* 276, 41263–41269. doi: 10.1074/jbc.M102381200

Slootweg, E., Roosien, J., Spiridon, L. N., Petrescu, A. J., Tameling, W., Joosten, M., et al. (2010). Nucleocytoplasmic distribution is required for activation of resistance by the potato NB-LRR receptor Rx1 and is balanced by its functional domains. *Plant Cell* 22, 4195–4215. doi: 10.1105/tpc.110.077537

Song, D. H., and Lee, J. O. (2012). Sensing of microbial molecular patterns by Toll-like receptors. *Immunol. Rev.* 250, 216–229. doi: 10.1111/j.1600-065X.2012.01167.x

Spassova, M. I., Klein-Lankhorst, R. M., Hille, J., Goldbach, R. W., Prins, M., Spassova, M. I., et al. (2001). The tomato gene Sw5 is a member of the coiled coil, nucleotide binding, leucine-rich repeat class of plant resistance genes and confers resistance to TSWV in tobacco. *Mol. Breed.* 7, 151–161. doi: 10.1023/A:1011363119763

Spoel, S. H., and Dong, X. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nat. Rev. Immunol.* 12, 89–100. doi: 10.1038/nri3141

Sun, Y., Li, L., Macho, A. P., Han, Z., Hu, Z., Zipfel, C., et al. (2013). Structural basis for flg22-induced activation of the *Arabidopsis* FLS2-BAK1 immune complex. *Science* 342, 624–628. doi: 10.1126/science.1243825

Takahashi, H., Miller, J., Nozaki, Y., Takeda, M., Shah, J., Hase, S., et al. (2002). RCY1, an *Arabidopsis thaliana* RPP8/HRT family resistance gene, conferring resistance to *Cucumber mosaic virus* requires salicylic acid, ethylene and a novel signal transduction mechanism. *Plant J.* 32, 655–667. doi: 10.1046/j.1365-313X.2002.01453.x

Takahashi, H., Suzuki, M., Natsuaki, K., Shigyo, T., Hino, K., Teraoka, T., et al. (2001). Mapping the virus and host genes involved in the resistance response in *Cucumber mosaic virus* infected *Arabidopsis thaliana*. *Plant Cell Physiol.* 42, 340–347. doi: 10.1093/pcp/pce039

Tameling, W. I., Nooijen, C., Ludwig, N., Boter, M., Slootweg, E., Goverse, A., et al. (2010). RanGAP2 mediates nucleocytoplasmic partitioning of the NB-LRR immune receptor Rx in the Solanaceae, thereby dictating Rx function. *Plant Cell* 22, 4176–4194. doi: 10.1105/tpc.110.077461

Tsuda, K., and Katagiri, F. (2010). Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Curr. Opin. Plant Biol.* 13, 459–465. doi: 10.1016/j.pbi.2010.04.006

Ueda, H., Yamaguchi, Y., and Sano, H. (2006). Direct interaction between the Tobacco mosaic virus helicase domain and the ATP-bound resistance protein, N factor during the hypersensitive response in tobacco plants. *Plant Mol. Biol.* 61, 31–45. doi: 10.1007/s11103-005-5817-8

Van der Hoorn, R. A., and Kamoun, S. (2008). From Guard to Decoy: a new model for perception of plant pathogen effectors. *Plant Cell* 20, 2009–2017. doi: 10.1105/tpc.108.060194

Verlaan, M. G., Hutton, S. F., Ibrahem, R. M., Kormelink, R., Visser, R. G., Scott, J. W., et al. (2013). The *Tomato Yellow Leaf Curl Virus* resistance genes Ty-1 and Ty-3 are allelic and code for DFDGD-class RNA-dependent RNA polymerases. *PLoS Genet.* 9:e1003399. doi: 10.1371/journal.pgen.1003399

Vidal, S., Cabrera, H., Andersson, R. A., Fredriksson, A., and Valkonen, J. P. (2002). Potato geneY-1 is an N gene homolog that confers cell death upon infection with potato virus Y. *Mol. Plant Microbe Interact.* 15, 717–727. doi: 10.1094/MPMI.2002.15.7.717

Wang, X., Goshe, M. B., Soderblom, E. J., Phinney, B. S., Kuchar, J. A., Li, J., et al. (2005). Identification and functional analysis of in vivo phosphorylation sites of the *Arabidopsis* BRASSINOSTEROID-INSENSITIVE1 receptor kinase. *Plant Cell* 17, 1685–1703. doi: 10.1105/tpc.105.031393

Wang, X., Kota, U., He, K., Blackburn, K., Li, J., Goshe, M. B., et al. (2008). Sequential transphosphorylation of the BRI1/BAK1 receptor kinase complex impacts early events in brassinosteroid signaling. *Dev. Cell* 15, 220–235. doi: 10.1016/j.devcel.2008.06.011

Wang, Y., Li, Z., Liu, D., Xu, J., Wei, X., Yan, L., et al. (2014). Assessment of BAK1 activity in different plant receptor-like kinase complexes by quantitative profiling of phosphorylation patterns. *J. Proteomics* 108, 484–493. doi: 10.1016/j.jprot.2014.06.009

Wen, R. H., Khatabi, B., Ashfield, T., Saghai Maroof, M. A., and Hajimorad, M. R. (2013). The HC-Pro and P3 cistrons of an avirulent *Soybean mosaic virus* are recognized by different resistance genes at the complex Rsv1 locus. *Mol. Plant Microbe Interact.* 26, 203–215. doi: 10.1094/MPMI-06-12-0156-R

Whitham, S., Dineshkumar, S. P., Choi, D., Hehl, R., Corr, C., and Baker, B. (1994). The product of the Tobacco mosaic virus resistance gene N: similarity to Toll and the Interleukin-1 receptor. *Cell* 78, 1101–1115. doi: 10.1016/0092-8674(94)90283-6

Whitham, S. A., Anderberg, R. J., Chisholm, S. T., and Carrington, J. C. (2000). *Arabidopsis* RTM2 gene is necessary for specific restriction of *Tobacco etch virus* and encodes an unusual small heat shock-like protein. *Plant Cell* 12, 569–582. doi: 10.2307/3871070

Win, J., Chaparro-Garcia, A., Belhaj, K., Saunders, D. G. O., Yoshida, K., Dong, S., et al. (2012). Effector biology of plant-associated organisms: concepts and perspectives. *Cold Spring Harb. Symp. Quant. Biol.* 77, 235–247. doi: 10.1101/sqb.2012.77.015933

Yamaji, Y., Maejima, K., Komatsu, K., Shiraishi, T., Okano, Y., Himeno, M., et al. (2012). Lectin-mediated resistance impairs plant virus infection at the cellular level. *Plant Cell* 24, 778–793. doi: 10.1105/tpc.111.093658

Yang, H., Gou, X., He, K., Xi, D., Du, J., Lin, H., et al. (2010). BAK1 and BKK1 in *Arabidopsis thaliana* confer reduced susceptibility to *turnip crinkle virus*. *Eur. J. Plant Pathol.* 127, 149–156. doi: 10.1007/s10658-010-9581-5

Yang, Z., Liu, L., Fang, H., Li, P., Xu, S., Cao, W., et al. (2016). Origin of the plant Tm-1-like gene via two independent horizontal transfer events and one gene fusion event. *Sci Rep.* 6:33691. doi: 10.1038/srep33691

Yang, Z. N., Ye, X. R., Molina, J., Roose, M. L., and Mirkov, T. E. (2003). Sequence analysis of a 282-kilobase region surrounding the *Citrus tristeza virus* resistance gene (Ctv) locus in *Poncirus trifoliata* L. Raf. *Plant Physiol.* 131, 482–492. doi: 10.1104/pp.011262

Yun, H. S., Bae, Y. H., Lee, Y. J., Chang, S. C., Kim, S. K., Li, J., et al. (2009). Analysis of phosphorylation of the BAK1/BRI1 complex in *Arabidopsis* reveals amino acid residues critical for receptor formation and activation of BR signaling. *Mol. Cells* 27, 183–190. doi: 10.1007/s10059-009-0023-1

Zhang, X. S., Choi, J. H., Heinz, J., and Chetty, C. S. (2006). Domain-specific positive selection contributes to the evolution of *Arabidopsis* leucine-rich repeat receptor-like kinase (LRR RLK) genes. *J. Mol. Evol.* 63, 612–621. doi: 10.1007/s00239-005-0187-z

Zhu, S., Jeong, R.-D., Venugopal, S. C., Lapchyk, L., Navarre, D., Kachroo, A., et al. (2011). SAG101 forms a ternary complex with EDS1 and PAD4 and is required for resistance signaling against *Turnip crinkle virus*. *PLoS Pathog.* 7:e1002318. doi: 10.1371/journal.ppat.1002318

Zipfel, C. (2014). Plant pattern-recognition receptors. *Trends Immunol.* 35, 345–351. doi: 10.1016/j.it.2014.05.004

Zorzatto, C., Machado, J. P. B., Lopes, K. V. G., Nascimento, K. J. T., Pereira, W. A., Brustolini, O. J., et al. (2015). NIK1-mediated translation suppression functions as a plant antiviral immunity mechanism. *Nature* 520, 679–682. doi: 10.1038/nature14171

Zvereva, A. S., and Pooggin, M. M. (2012). Silencing and innate immunity in plant defense against viral and non-viral pathogens. *Viruses* 4, 2578–2597. doi: 10.3390/v4112578

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Genetics and Molecular Biology, 40, 1, xxx-xxx (2017)

DOI: http://dx.doi.org/10.1590/1678-4685-GMB-2016-0092

Review Article

Translational control in plant antiviral immunity

João Paulo B. Machado[1*], Iara P. Calil[1*], Anésia A. Santos[1] and Elizabeth P.B. Fontes[1]

[1]*Department of Biochemistry and Molecular Biology, BIOAGRO, National Institute of Science and Technology in Plant-Pest Interactions, Universidade Federal de Viçosa, 36571.000, Viçosa, MG, Brazil.*

Abstract

Due to the limited coding capacity of viral genomes, plant viruses depend extensively on the host cell machinery to support the viral life cycle and, thereby, interact with a large number of host proteins during infection. Within this context, as plant viruses do not harbor translation-required components, they have developed several strategies to subvert the host protein synthesis machinery to produce rapidly and efficiently the viral proteins. As a countermeasure against infection, plants have evolved defense mechanisms that impair viral infections. Among them, the host-mediated translational suppression has been characterized as an efficient mean to restrict infection. To specifically suppress translation of viral mRNAs, plants can deploy susceptible recessive resistance genes, which encode translation initiation factors from the eIF4E and eIF4G family and are required for viral mRNA translation and multiplication. Additionally, recent evidence has demonstrated that, alternatively to the cleavage of viral RNA targets, host cells can suppress viral protein translation to silence viral RNA. Finally, a novel strategy of plant antiviral defense based on suppression of host global translation, which is mediated by the transmembrane immune receptor NIK1 (nuclear shuttle protein (NSP)-Interacting Kinase1), is discussed in this review.

Keywords: Translation suppression, recessive resistance genes, Argonaute, NSP-Interacting Kinase, NIK.

Received: April 06, 2016; Accepted: September 27, 2016.

Introduction

Due to their sessile nature, plants are constantly exposed to extreme adverse conditions that affect negatively their growth and development, thereby resulting in considerable yield losses worldwide. Among the biotic factors, virus infections are one of the most prevalent agricultural constraints as they often suppress the plant defenses and severely limit productivity of relevant crops, representing a serious threat to global food security. As obligatory intracellular parasites, plant viruses depend on the host cell machinery to replicate their genome, express their genes and invade their hosts. Thus, in order to establish a productive infection, compatible interactions between viral and host factors must occur, from the expression and replication of the viral genome until the cell-to-cell movement and long distance translocation through the vascular system of viral particles. In addition to providing basic compatibility, interactions between viral and host proteins are necessary to modulate the viral infection, preventing or neutralizing the plant defense mechanisms.

Send correspondence to Elizabeth P B Fontes. Department of Biochemistry and Molecular Biology, BIOAGRO, National Institute of Science and Technology in Plant-Pest Interactions, Universidade Federal de Viçosa, Av. PH Rolfs s/n, 36571.000, Viçosa, MG, Brazil. Email: bbfontes@ufv.br

*These authors contributed equally to this work.

Plants deploy several strategies to defend themselves against viral infections; the best characterized are the expression of resistance genes and RNA silencing (Nicaise, 2014). Naturally occurring resistance genes, which show dominant or recessive inheritance, bestow an efficient barrier to viral infection (Robaglia and Caranta, 2006). Most of the dominant resistance genes (R genes) identified in plant–virus interactions belong to the nucleotide binding site leucine-rich repeat (NBS-LRR) class, which specifically recognize viral avirulence (avr) gene products (De Ronde *et al.*, 2014; Galvez *et al.*, 2014; Nicaise, 2014). Frequently, the R protein activation elicits a hypersensitive response (HR), which is often associated with programmed cell death of infected and adjacent cells, confining the pathogen within the local site of infection (Gururani *et al.*, 2012; Galvez *et al.*, 2014). The downstream events of R protein activation might be also associated with other signals like influx of Ca^{2+} ions from the extracellular space and/or anion flux, MAPK-mediated signaling, production of reactive oxygen species (ROS), salicylic acid (SA) accumulation, extensive transcriptional reprogramming and activation of defense responses (Gururani *et al.*, 2012; Nicaise, 2014). Additionally to dominant R gene–related resistance responses, recessive resistance has been commonly reported in viral systems as another evolving strategy to impair virus infection (Kang *et al.*, 2005b). Instead of triggering typical defense responses, like the hypersensi-

tive response, most recessive mutations lead to a non-permissive environment due to the lack of appropriate host factors, which are required for the viruses to complete their biological cycle (Ritzenthaler, 2005; Galvez *et al.*, 2014). In contrast to dominant resistance, recessive resistance seems to be more durable because the viruses can only overcome the host resistance response by adapting themselves to the missing factors (Ritzenthaler, 2005; Truniger and Aranda, 2009). Because viruses do not encode translational functions and depend exclusively on the host cell machinery to synthesize the viral proteins, it is not surprising that a large number of recessive resistance genes have been mapped to mutations in translation initiation factors (eIFs) belonging to the eIF4E and eIF4G family or their isoforms eIFiso4E and eIFiso4G (Truniger and Aranda, 2009; Wang and Krishnaswamy, 2012; Julio *et al.*, 2015). In general, these mutations prevent the interactions between host factors and viral RNAs and/or proteins, which otherwise would recruit the host apparatus of translation for the synthesis of viral proteins. The partial functional redundancy of isoforms from the eIF4E and eIF4G families allows loss-of-function mutations of one isoform to provide virus resistance without compromising the general growth performance of the plant.

RNA silencing also represents a well-documented antiviral mechanism in plants (Ding, 2010; Pumplin and Voinnet, 2013; Csorba *et al.*, 2015; Ghoshal and Sanfaçon, 2015). Viral RNAs can be addressed for degradation via the endonucleolytic activity of argonaute (AGO), the catalytic component of the RNA-induced silencing complex (RISC; Mallory and Vaucheret, 2010). In addition to the endonucleolytic cleavage, recent evidence has demonstrated that the mechanism of antiviral RNA silencing also operates by suppressing viral mRNA translation (Ghoshal and Sanfaçon, 2015). As a virulence strategy, plant viruses have evolved mechanisms to prevent RNA silencing-mediated defense, predominantly by synthesizing silencing suppressors (Bologna and Voinnet, 2014; Carbonell and Carrington, 2015).

In addition to translational defense mechanisms based on recessive resistance and RNA silencing, a novel strategy of translational suppression in plant defense against DNA viruses (begomoviruses) has recently emerged as a new paradigm of antiviral defenses in plants. In this case, the activation of the transmembrane immune receptor NIK1 [nuclear shuttle protein (NSP)-interacting kinase 1] promotes the down-regulation of translational machinery-associated genes, culminating in the inhibition of viral and host mRNAs translation, which causes an increase in tolerance to begomoviruses (Zorzatto *et al.*, 2015). These defense strategies against viruses strengthen the argument that the inhibition of translation of viral proteins or their capacity to interact with translational factors offer promising alternatives to control viruses in plants.

Recessive resistance genes in translational control

Plants respond to pathogens through an elaborate network of genetic interactions and the outcome of these interactions can result in disease or resistance. Among the plant resistance genes, the recessive ones play relevant roles in plant defense against viruses and comprise about one-half of known antiviral resistance genes (Sanfaçon, 2015). Recessive resistance is frequently associated with the lack of host factors necessary for the completion of the virus biological cycle (Galvez *et al.*, 2014). In order to achieve a successful infection, the viruses not only need unrestricted access to the host translation machinery to synthesize their proteins, but they also need to suppress host innate defenses, which may act to impair the protein production capacity of the infected cells (Walsh and Mohr, 2011). The majority of recessive genes involved in plant–virus interactions encode eukaryotic translation initiation factors (eIFs) of the 4E or 4G family, mainly eIF4E, eIF4G and their isoforms (Kang *et al.*, 2005b; Truniger and Aranda, 2009; Wang and Krishnaswamy, 2012). The involvement of eIF4E and eIF4G was firstly reported in potyvirus infection and subsequently expanded to include other plant virus families, such as bymoviruses, cucumoviruses, ipomoviruses, sobemoviruses, carmoviruses, and waikiviruses, suggesting that they contribute to a broad mechanism of plant susceptibility to viruses (Nicaise, 2014). In eukaryotes, mRNA translation is predominantly cap-dependent and involves the assembly of an mRNA-protein complex by different eIFs (Aitken and Lorsch, 2012; Hinnebusch, 2014). eIF4E is a cap-binding protein involved in the initiation of translation, being part of the protein complex known as eIF4F, which also contains eIF4G and the DEAD-box RNA helicase eIF4A. The eIF4F complex, comprising eIF4G, eIF4E and eF4A, binds poly(A)-binding protein (PABP) and eIF3 (Jackson *et al.*, 2010; Sanfaçon, 2015). In contrast to other eukaryotes, plants possess a second form of eIF4F, named eIF(iso)4F, which includes eIF(iso)4E and eIF(iso)4G (Patrick and Browning, 2012; Sanfaçon, 2015). eIF(iso)4F has complementary activities with eIF4F, but their respective components are differentially expressed, suggesting that they may also display distinct functions (Wang, 2015). Several resistance genes encoding a mutated form of eIF4E or eIF(iso)4E proteins have been shown to mediate resistance against viral infection in a range of plant/virus interactions. These include sbm-1 against *Pea seed-borne mosaic viru*s (PsbMV) and cyv2 against *Clover yellow vein virus* (ClYVV) in pea; mo1 (1), mol1 (2) in lettuce against *Lettuce mosaic virus* (LMV); pvr1, pvr2 and pvr6 in pepper against *Tobacco etch virus* (TEV), *Potato virus Y* (PVY), *Pepper veinal mottle virus* (PVMV); rym4 and rym5 in barley against *Barley yellow mosaic virus* (BaYMV); nsv in melon against *Melon necrotic spot virus* (MNSV); pot-1 in tomato against PVY

and TEV; lsp1 in Arabidopsis against *Turnip mosaic virus* (TuMV) and TEV (Lellis *et al.*, 2002; Ruffel *et al.*, 2002, 2005, 2006; Nicaise *et al.*, 2003; Gao *et al.*, 2004a,b; Kang *et al.*, 2005a; Stein *et al.*, 2005; Albar *et al.*, 2006; Nieto *et al.*, 2006; Andrade *et al.*, 2009). Additionally, genes encoding a mutated form of eIF4G or its defective isoforms, such as rymv1 and tsv1, are responsible for resistance to *Rice yellow mottle virus* (RYMV) and *Rice tungro spherical virus* (RTSV) in rice (Albar *et al.*, 2006; Lee *et al.*, 2010). The cum1 and cum2 mutations, coding for translation initiation factors 4E and 4G, respectively, inhibit *Cucumber mosaic virus* (CMV*)* multiplication in Arabidopsis and *Turnip crinkle virus* (TCV) in Arabidopsis (Yoshii *et al.*, 2004).

An important step on the elucidation of the molecular nature of recessive resistance was the identification of VPg (genome-linked viral protein) from several potyviruses as an interacting partner of the translation initiation factor 4E (eIF4E) or its isoform eIF(iso)4E in yeast two-hybrid and *in vitro* binding assays (Figure 1) (Wittmann *et al.*, 1997; Léonard *et al.*, 2000; Schaad *et al.*, 2000; Lellis *et al.*, 2002; Robaglia and Caranta, 2006). Mutations in VPg, which disrupt VPg-eIF(iso)4E interaction, impair viral infection *in planta* (Léonard *et al.*, 2000). The VPg protein may act mimicking the 5'-cap structure of messenger RNAs and recruiting the translation complex for viral genome translation through its specific interaction with eIF4E/eIF(iso)4E (Michon *et al.*, 2006; Wang, 2015). Thus, VPg protein facilitates viral RNA translation by competing with the eIF4E/eIFiso4E cap binding activity and enhancing the affinity of eIF4E/iso4E for viral RNAs *in vitro.* (Plante *et al.*, 2004; Khan *et al.*, 2008). The host factor eIF4G/eIF(iso)4G is also important for a potyvirus infection as it enhances VPg-eIF4E/eIF(iso)4E interactions (Nicaise *et al.*, 2007). The VPg protein of *Rice yellow mottle virus* (RYMV) binds directly to eIFiso4G, rather than to eIF4E isoforms (Hebrard *et al.*, 2010). These observations suggest that VPg recruits the whole eIF4F complex, possibly for the translation of viral RNAs (Sanfaçon, 2015).

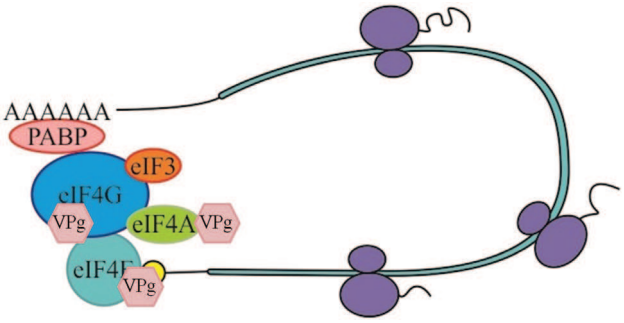

Figure 1 - Host translation factors targeted by genome-linked viral protein (VPg) in canonical eukaryotic translation. VPg recruits the translation initiation complex for viral genome translation through its specific interaction with eIF4E/eIF(iso)4E. VPg also binds to eIF4G/eIF(iso)4G, which may act enhancing VPg-eIF4E/eIF(iso)4E interactions. Potyvirus VPg protein interacts with DEAD-box RNA helicase-like proteins closely related to *A. thaliana* eIF4A genes. RNA helicase, as part of the eIF4F translation complex, might be also involved in viral genome replication.

Although the VPg-eIF4E/iso4E complex may support viral RNA translation in some potyvirus-host interactions, it probably contributes to other steps of the infection. Accordingly, *Tobacco etch virus*(TEV) has been demonstrated to depend on eIF(iso)4E for its systemic spread in Arabidopsis, suggesting a role of eIF(iso)4E in viral movement (Contreras-Paredes *et al.*, 2013). This interpretation was consistent with the observation that inactivation and overexpression of the *eIF(iso)4E* gene did not affect global cellular or viral translation and, in the null mutant, viral replication was still observed. These results indicate that, for the TEV-Arabidopsis system, eIF(iso)4E is not required for viral translation and replication in the viral infective cycle, but is required for viral movement, as TEV systemic spread was completely abolished in the eif(iso)4f knockout lines.

Other components of the host translation machinery, such as eEF1A and PABP, are found in the virus translation/replication complex (Beauchemin and Laliberte, 2007; Thivierge *et al.*, 2008; Wang, 2015). eEF1A, eEF1B and an eIF3 subunit are required for TMV infection (Osman and Buck, 1997; Yamaji *et al.*, 2006; Hwang *et al.*, 2013). The RNA of *Turnip yellow mosaic virus* (TYMV) possesses a tRNA-like structure at 3' UTR instead of a poly(A) tail, which works as a translation enhancer of the viral RNA. This region interacts with the host factor eEF1A and seems to regulate viral replication (Matsuda and Dreher, 2004; Sanfaçon, 2015). The ribosomal protein P0 has been correlated with viral RNA translation in *Potato virus A* (PVA) infection (Hafren *et al.*, 2013). Huang *et al.* (2010) have reported the identification of two VPg-interacting plant DEAD-box-containing RNA helicase-like proteins, AtRH8 from Arabidopsis and PpDDXL from peach (Figure 1). These proteins share sequence homology with eIF4A, a component of the eIF4F multiprotein complex. AtRH8 is not required for plant growth and development, but is necessary for viral infection. Arabidopsis *atrh8* mutant plants were resistant to both plant potyvirus PPV and TuMV.

Additionally to their roles in the host translation of mRNAs, translation factors play other biological functions that might be exploited by viruses. eIF4E has been shown to accumulate in nuclear bodies, where it is involved in the export of a subset of mRNAs containing a structure known as a 4E-sensitivity element (Goodfellow and Roberts, 2008, Truniger and Aranda, 2009). In this context, it would be possible that VPg acts in the nucleus suppressing eIF4E-mediated mRNA exportation to the cytoplasm (Wang and Krishnaswamy, 2012). This hypothesis is supported by the observation that VPg inhibits the translation of the capped mRNAs (Khan *et al.*, 2008; Eskelin *et al.*, 2011). eIF4E–VPg complex may be also involved in the suppression of RNA silencing, in which VPg acts as an accessory factor for HC-Pro (silencing suppressor protein)

and promotes disturbance of siRNA and microRNA processing in the nucleus (Kasschau and Carrington, 1998; Rajamäki and Valkonen, 2009).

Antiviral roles of plant argonautes in translation repression and virus countermeasures

Argonautes (AGOs) are the effector proteins functioning in eukaryotic RNA silencing pathways (Carbonell and Carrington, 2015; Fang and Qi, 2016), a sequence-specific process that serves two main functions: regulation of gene expression and defense against pathogens (Mandadi and Scholthof, 2013; Bologna and Voinnet, 2014; Nicaise, 2014; Zhang *et al.*, 2015; Wang and Chekanova, 2016). RNA silencing is triggered by the presence of double-stranded RNA (dsRNA), which is processed into small RNA (sRNA) molecules of 21–24 nucleotide (nt) by RNase III-type enzymes called Dicer, or Dicer-like (DCL) in plants (Figure 2; Krol *et al.*, 2010). Upon processing, one strand of the sRNA duplexes is incorporated into RNA-induced silencing complexes (RISCs), whose key catalytic component corresponds to one member of the AGO protein family. Once integrated into the RISC, sRNAs guide the sequence-specific inactivation of the targeted RNA or DNA (Kamthan *et al.*, 2015). The mechanisms of action of AGO/sRNA complexes at the RNA level include mRNA cleavage or translational repression (post-transcriptional gene silencing, PTGS; Figure 2), whereas, at the DNA level, they involve DNA and/or histone methylation and subsequent transcriptional gene silencing (TGS) (Martinez de Alba *et al.*, 2013).

Translational repression guided by sRNAs has been best studied in fly and mammalian cells and is mediated by imperfect base pairing of microRNAs (miRNAs) to target mRNAs (Wilczynska and Bushell, 2015). In animal cells, miRNAs normally bind to the 3'-untranslated region (3'UTR) of target mRNAs and direct not only translation repression but also mRNA destabilization, which are initiated by deadenylation/decapping enzymes. Both processes require association of AGO proteins with proteins containing glycine-tryptophan (GW/WG) motifs, such as members of the GW182 family (Fukaya and Tomari, 2012; Pfaff and Meister, 2013). In plants, early studies have shown that miRNAs display a high degree of sequence complementarity to their target mRNAs and they guide cleavage of target RNAs through endonucleolytic activity of AGO1 (Tang *et al.*, 2003; Baumberger and Baulcombe, 2005). This led to the assumption that RNA cleavage is the major mode of action of plant miRNAs (Jones-Rhoades *et al.*, 2006; Huntzinger and Izaurralde 2011). However, recent reports suggest that plant miRNAs mediate not only the cleavage of the target but also a concurrent translation repression (Brodersen *et al.*, 2008; Lanet *et al.*, 2009; Yang *et al.*, 2012; Iwakawa and Tomari 2013; Li *et al.*, 2013). Evidence that miRNAs repress translation in plants emerged from the

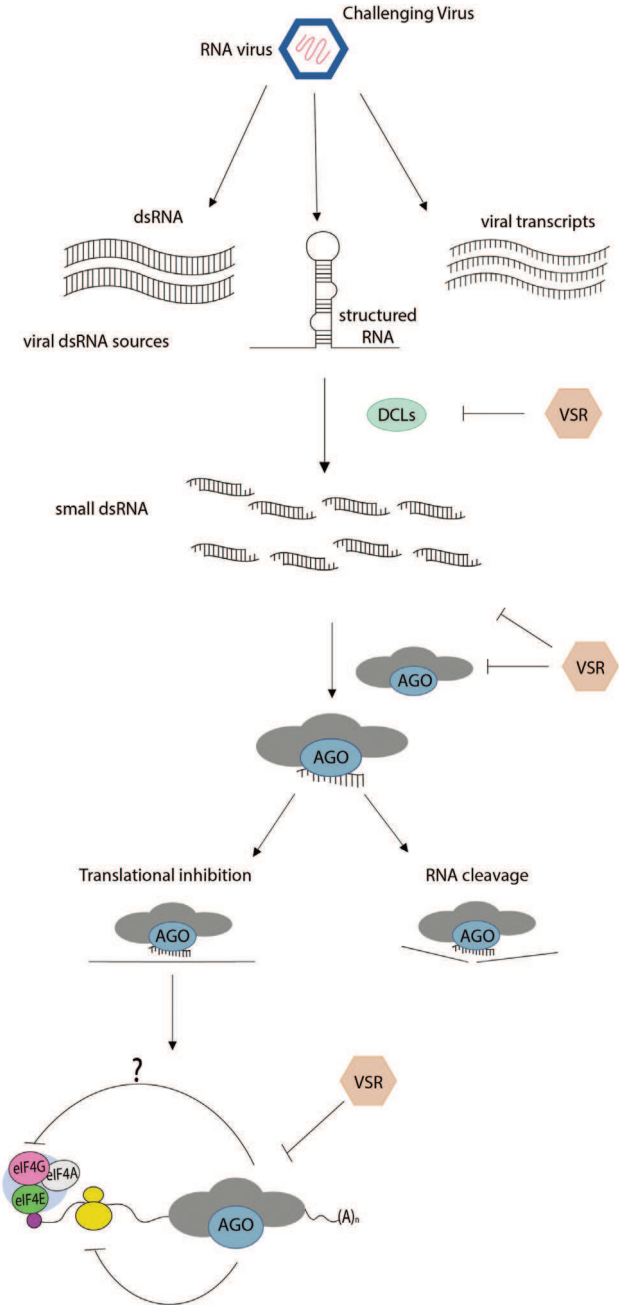

Figure 2 - An integrating overview of RNA silencing and AGO-mediated translational repression of mRNA targets. RNA silencing is launched by viral double-stranded RNAs (dsRNAs) from different sources, which are processed into small interfering RNA molecules of 21–24 nucleotide (nt) by Dicer-like (DCL) proteins. Subsequently one strand of the siRNA duplexes is loaded into RNA-induced silencing complexes (RISCs) harboring an argonaute (AGO) effector protein. In light of post-transcriptional gene silencing (PTGS), AGO/sRNA complexes trigger viral RNA cleavage or translational repression. Although unclear, translation repression directed by sRNAs in plants seems to rely on AGO activity, which may act targeting ribosome assembly, interfering with 48S initiation complex formation, or translational initiation factors (eIFs). The AGO-RISC complex is further capable of repressing translation by preventing translation elongation or ribosomal recruitment. Viral suppressors of RNA silencing (VSRs) interfere in multiple steps of the antiviral RNA silencing pathway, including dicing inhibition, viral RNA loading, AGO inactivation and suppression of its translational inhibitory activity.

examination of protein accumulation from miRNA-targeted genes (Aukerman and Sakai, 2003; Chen, 2004; Gandikota *et al.*, 2007). In these studies, plant miRNAs were found to exert disproportionate effects on target gene expression at mRNA versus protein levels. Furthermore, mutations in a number of genes, including the P body component VARICOSE (VCS) and ALTERED MERISTEM PROGRAM1 (AMP1), an integral membrane protein associated with endoplasmic reticulum (ER) and AGO1, impair miRNA-mediated target repression at the protein but not at the mRNA level (Brodersen *et al.*, 2008; Li *et al.*, 2013). Additional evidence for the miRNA-mediated translational repression in plants came from the observation that AGO1 and several miRNA are associated with polysomes (Llave *et al.*, 2002).

AGO1, AGO2 and AGO10 have been implicated in translation repression (Brodersen *et al.*, 2008; Lanet *et al.*, 2009; Fátyol *et al.*, 2016). Mutations in AGO1 and AGO10 genes impair miRNA-mediated target repression at the protein but not at the mRNA level (Brodersen *et al.*, 2008). Furthermore, by using an *in vitro* system prepared from plant cultured cells, AtAGO1 has been shown to have the ability to repress translation initiation even when the cleavage of mRNA targets was blocked by introducing central mismatches in the miRNA-target pairing region or mutation of the catalytic core of AtAGO1 (Iwakawa and Tomari, 2013). Recently, Fátyol *et al.* (2016) showed, using a sensitive transient *in vivo* reporter system, that AGO2 is capable of exerting translational repression in various miRNA target site constellations (Open reading frame – ORF, 3'UTR).

The mechanism of AGO-mediated translational repression in plant cells is less clear than in animal cells. Although plant cells apparently lack orthologs of GW182 (Huntzinger and Izaurralde 2011), which are proteins containing GW/WG motifs required for AGO-mediated translation suppression in animal cells (Fukaya and Tomari, 2012; Pfaff and Meister, 2013), the Arabidopsis SUO protein (a large protein with two C-terminal GW motifs) is required for miRNA directed translation repression and may serve as a functional analog of the GW182 proteins (Yang *et al.*, 2012). In line with the notion that plants likely lack orthologs of GW182, deadenylation of the mRNAs has not been observed in tobacco cell lysates (Iwakawa and Tomari, 2013). Binding of miRNAs is not only restricted to the 3'UTR of the mRNAs but also occurs in the 5'UTR and even in the ORF (Iwakawa and Tomari, 2013; Fátyol *et al.*, 2016). A detailed *in vitro* study revealed that several distinct types of translation repression could be mediated by plant miRNAs depending on the position of the target sites (Iwakawa and Tomari, 2013). When target sites reside in the 3'UTR of the mRNAs, AtAGO1-RISC is capable of repressing translation initiation by interfering with 48S initiation complex formation, a mechanism similar to that observed in animal cells (Figure 2). Binding of miRNAs to targets within the ORF functions differently by preventing translation elongation (Iwakawa and Tomari, 2013). When extensively complementary target sites reside in the 5'UTR, AtAGO1-RISC can sterically hinder ribosomal recruitment (Iwakawa and Tomari, 2013).

Antiviral RNA silencing is triggered by highly structured viral ssRNA or dsRNA, which are recognized and processed by DCLs to produce viral small interfering RNAs (vsiRNAs) that are subsequently incorporated into antiviral RISCs (Mallory and Vaucheret, 2010). Antiviral AGOs associate with vsiRNAs and target complementary viral RNAs for degradation through endonucleolytic cleavage (slicing) and/or for translational arrest, transcriptionally repress complementary viral DNA through hypermethylation, or regulate host gene expression to promote defense (Szittya and Burgyan, 2013). These processes can result in a phenomenon known as recovery, whereby the plant silences viral gene expression and recovers from viral symptoms. The targeting of viral RNAs is thought to largely involve RNA cleavage. Nevertheless, recent studies have identified AGO-mediated translation repression as an additional RNA silencing mechanism against plant viruses (Bhattacharjee *et al.*, 2009; Ghoshal and Sanfaçon, 2014; Karran and Sanfaçon, 2014; Ma *et al.*, 2015).

Translational repression of viral mRNAs was first observed in association with the defense response activated by the interaction between a dominant resistance gene and a viral elicitor (Bhattacharjee *et al.*, 2009). Co-expression of a resistance protein with nucleotide-binding (NB) and leucine-rich repeat (LRR) domain (NB–LRR) and its cognate viral effector results in an antiviral response that inhibits the translation of virus-encoded proteins in *Nicotiana benthamiana* (Bhattacharjee *et al.*, 2009). Both the translational repression of viral transcripts and NB-LRR-mediated virus resistance were impaired by the downregulation of Argonaute 4-like genes. These results suggest that AGO proteins are involved in the specific translational control of viral transcripts in virus resistance mediated by NB–LRR proteins (Bhattacharjee *et al.*, 2009). Translation inhibition was also observed in a study with *tobacco rattle virus* (TRV) in Arabidopsis (Ma *et al.*, 2015). In this study, recovered plants showed reduced association of TRV RNAs with ribosomes and an increase in the formation of RNA processing bodies (PBs). Another example of AGO-dependent translational repression mechanism was observed in *N. benthamiana* plants infected with *Tomato ringspot virus* (ToRSV) (Ghoshal and Sanfaçon, 2014). In this interaction, symptom recovery follows an initial symptomatic systemic infection. These authors also showed that the recovery of ToRSV-infected plants is associated with a reduction in the steady-state levels of viral proteins and decreased translation of the corresponding viral RNA. *In vivo* labeling experiments revealed efficient synthesis of the RNA2-encoded coat protein (CP) early in infection, but reduced RNA2 translation later in infection. Additionally,

neither recovery nor the reduction of RNA2 translation were observed in plants silenced for AGO1, suggesting that AGO1 plays a role in the translational repression mechanism targeting ToRSV (Ghoshal and Sanfaçon 2014).

As a counter-defense strategy against antiviral RNA silencing mechanism, most plant viruses have evolved specialized proteins known as viral suppressors of RNA silencing (VSRs), which disrupt various steps of the silencing pathway (Burgyán and Havelda 2011; Pumplin and Voinnet, 2013; Csorba *et al.*, 2015). AGO proteins are preferred targets of VSRs at multiple levels (Figure 2). Among the well-characterized VSRs, some of them (e.g., the tombusvirus p19 and the potyvirus HC-Pro proteins) directly bind and sequester vsiRNA duplexes away from antiviral AGOs, preventing their loading into the RISC (Vargason *et al.*, 2003; Ye *et al.*, 2003; Csorba *et al.*, 2015; Garcia-Ruiz *et al.*, 2015). VSRs can also prevent AGO association with vsiRNAs by promoting AGO degradation, as observed for polerovirus and enamovirus. P0 proteins destabilize AGO1 through an F-box-like domain and induce subsequent degradation through the autophagy pathway (Baumberger *et al.*, 2007; Bortolamiol *et al.*, 2007; Csorba *et al.*, 2010; Derrien *et al.*, 2012; Fusaro *et al.*, 2012). Likewise, the silencing suppressor P25 of *Potato virus X* interacts with AGO1 and mediates its degradation through the proteasome pathway (Chiu *et al.*, 2010).

VSRs can also block vsiRNA-programmed AGOs. The cucumovirus 2b protein interacts directly with AGO1, and this interaction occurs primarily on one surface of the PAZ domain and part of the PIWI domain of AGO1 (Zhang *et al.*, 2006). Consistent with this interaction, 2b specifically inhibits the AGO1 slicing activity in RISC (Zhang *et al.*, 2006). Some VSRs, including the ipomovirus P1, carmovirus p38 and nepovirus CP proteins, contain WG/GW motifs that mimic AGO1-interacting cellular proteins (Azevedo *et al.*, 2010; Giner *et al.*, 2010; Szabo *et al.*, 2012; Zhang *et al.*, 2012; Karran and Sanfaçon, 2014). P1 protein from *Sweet potato mild mottle virus* (SPMMV) targets loaded AGO1 and inhibits the si/miRNA-programmed RISC activity. The suppressor/binding activities are localized at the N-terminal half of P1, a region containing three WG/GW motifs (Giner *et al.*, 2010). The importance of the Glycine-Tryptophan (GW) motifs in AGO1 binding and suppression activity was further demonstrated when *Sweet potato feathery mottle virus* (SPFMV) P1, which did not have any silencing suppressor activity, was converted into a VSR by including two additional WG/GW motifs (Szabo *et al.*, 2012). P38 protein from *Turnip crinkle virus* (TCV) physically interacts through GW repetitive motifs with unloaded Arabidopsis AGO1 (Azevedo *et al.*, 2010) or AGO2 (Zhang *et al.*, 2012), suppressing RNA silencing. Another example of VSR that acts through interaction with AGO1 in a WG motif-dependent manner is ToRSV CP protein (Karran and Sanfaçon, 2014). The WG motif within the CP is required for silencing suppression, AGO1 binding, CP mediated AGO1 degradation, suggesting that the ToRSV CP acts as an AGO-hook protein and competes for AGO binding with a plant cellular GW/WG protein involved in translation repression (Karran and Sanfaçon, 2014).

The translational control branch of the NIK-mediated antiviral signaling

The immune receptor NIK1 [nuclear shuttle protein (NSP)-interacting kinase 1] has a remarkable role in the defense response against begomoviruses. It belongs to the receptor-like kinase (RLK) family of plant receptors, and it was first identified as a virulence target of the begomovirus nuclear shuttle protein (NSP) (Fontes *et al.*, 2004). NSP is encoded by the component B, DNA-B, of bipartite begomoviruses (*Geminiviridae* family) that also encodes the movement protein (MP), both being viral proteins required for systemic infection (Hanley-Bowdoin *et al.*, 2013). The proteins required for replication (Rep and REn), transactivation of viral genes (TrAP), the suppression of RNAi defense functions (TrAP and AC4) and encapsidation of viral DNA (CP) are encoded by the other genomic component, DNA-A. Begomoviruses replicate their genome in the nuclei of infected plants via rolling circle replication. NSP facilitates the traffic of viral DNA from the nucleus to the cytoplasm and acts in concert with MP to move the viral DNA to the adjacent, uninfected cells (Hanley-Bowdoin *et al.*, 2013).

Virus propagation is usually restricted by the activation of the small interfering RNA (siRNA) antiviral machinery and/or salicylic acid (SA) signaling pathway (Nicaise, 2014). In the case of begomoviruses, it has been shown that in addition to encoding suppressors for siRNA-mediated defenses, these viruses enhance their pathogenicity in susceptible hosts by suppressing the antiviral activity of the transmembrane receptor NIK1 by the viral NSP (Fontes *et al.*, 2004; Santos *et al.*, 2009; Brustolini *et al.*, 2015).

Within the RLK family, NIKs receptors (NIK1, NIK2 and NIK3) belong to the subfamily II of leucine-rich repeat (LRR)-RLKs, designated LRRII-RLK group (Shiu and Bleecker, 2001; Dievart and Clark, 2004). NIK1 was identified through two-hybrid screening using the viral protein NSP as bait (Fontes *et al.*, 2004; Mariano *et al.*, 2004). The NSP-NIK1 interaction was further demonstrated by *in vitro* GST pull-down assays and confirmed *in planta* through bimolecular fluorescence complementation (BiFC) assays (Fontes *et al.*, 2004; Brustolini *et al.*, 2015). The NSP–NIK interaction is conserved among begomovirus NSPs and NIK homologues from different hosts. NIK homologs from Arabidopsis, tomato and soybean interact with NSP from *Cabbage leaf curl virus* (CaLCuV) and from tomato-infecting begomoviruses, such as *Tomato golden mosaic virus* (TGMV), *Tomato crinkle leaf yellow virus* (TCrLYV) and *Tomato yellow spot virus* (ToYSV) (Fontes *et al.*,

2004; Mariano *et al.*, 2004; Sakamoto *et al.*, 2012). Using the two-hybrid system in yeast, the NSP-binding site was mapped to an 80 amino acid stretch of the kinase domain (positions 422–502) of NIK1 that encompasses the putative active site for Ser/Thr kinases (subdomain VIb–HrDvKssNxLLD) and the activation loop (subdomain VII–DFGAk/rx, plus subdomain VIII–GtxGyiaPEY) (Fontes *et al.*, 2004).

NSP from CaLCuV acts as a virulence factor to suppress the kinase activity of transmembrane receptor NIKs, suggesting that NIK is involved in antiviral defense response (Fontes *et al.*, 2004). Several lines of evidence further support a NIK role in antiviral defense. Firstly, loss of *NIK* function in Arabidopsis is linked to an enhanced susceptibility phenotype to infection by a coat protein-less mutant of CaLCuV (Fontes *et al.*, 2004; Carvalho *et al.*, 2008; Santos *et al.*, 2009). In addition, overexpression of *NIK1* from Arabidopsis in tomato plants attenuates symptom development and delays ToYSV infection (Carvalho *et al.*, 2008). Finally, mutations in the activation loop (A-loop) of NIK1 that block its autophosphorylation activity also impair the capacity of NIK1 to elicit a response against begomoviruses (Santos *et al.*, 2009).

As Ser/Thr kinase receptors, NIKs contain all of the 11 conserved subdomains of protein kinases, in addition to specific signatures of serine/threonine kinases in subdomains VIb and VIII (Hanks *et al.*, 1988), including the A-loop, region highly conserved among members of the LRRII-RLK subfamily and other members of the extended LRR-RLK family (Hubbard, 1997; Bellon *et al.*, 1999; Biondi *et al.*, 2002; Yang *et al.*, 2002; Kornev *et al.*, 2006). NIK1 kinase activity has been shown to be dependent on the phosphorylation status of the A-loop (Fontes *et al.*, 2004; Carvalho *et al.*, 2008; Santos *et al.*, 2009). NIK1 is phosphorylated *in vitro* at the conserved positions Thr-474 and Thr-469, and mutations within the A-loop interfere in the NIK1 capacity of autophosphorylation (Santos *et al.*, 2009). Replacement of Thr474 with alanine (T474A) strongly inhibits the autophosphorylation activity. This activity is completely abolished by removing the conserved Gly-473 residue in the T474A mutant to valine (G473V/T474A). In contrast, replacement of Thr-474 with a phosphomimetic aspartate residue increases autophosphorylation activity and results in constitutive activation of a NIK1 mutant receptor that it is no longer inhibited by the begomovirus NSP (Santos *et al.*, 2009). The biological relevance of these findings has been certified by *in vivo* complementation assays. Ectopic expression of T474A defective kinase or G473A/T474A inactive kinase does not complement the *nik1* loss-of-function defect, demonstrating that Thr-474 autophosphorylation is required to transduce a defense response to begomoviruses (Santos *et al.*, 2009). In contrast, ectopic expression of the Arabidopsis phosphomimetic T474D mutant in tomato transgenic lines confers higher level of tolerance to tomato-infecting begomoviruses than expression of an intact *NIK1* receptor (Brustolini *et al.*, 2015). Collectively, these results implicate the phosphorylation at the essential Thr-474 residue within the A-loop as a key regulatory mechanism for NIK activation.

The ribosomal protein L10 (RPL10), isolated through two-hybrid screening by its capacity to bind to the kinase domain of NIK1 (Rocha *et al.*, 2008), acts as a downstream effector of the NIK-mediated antiviral response (Carvalho *et al.*, 2008). Consistent with an RPL10 role in antiviral defense, loss of *RPL10* function recapitulated the *nik1* enhanced susceptibility phenotype to begomovirus infection, as the *rpl10* knockout lines developed similar severe symptoms and displayed similar infection rate as *nik1* (Carvalho *et al.*, 2008; Rocha *et al.*, 2008). The RPL10 protein from Arabidopsis shows sequence similarity with the human L10 protein, also called the QM, and, like QM, displays nucleocytoplasmatic shuttling. In fact, RPL10 is localized in the cytoplasm, but is phosphorylated and redirected to the nucleus by co-expression with NIK1 (Carvalho *et al.*, 2008). Although RPL10 binds to NIK1 *in vitro* and *in vivo*, it is not efficiently phosphorylated by NIK1 *in vitro* and may not serve as a direct NIK1 substrate *in vivo*. Nevertheless, several lines of evidence indicate that the nucleocytoplasmic shuttling of RPL10 is dependent on the phosphorylation status and kinase activity of NIK1. While the defective T474A or the inactive G473A/T474A NIK1 mutants failed to redirect RPL10 to the nuclei of co-transfected cells, expression of the hyperactive T474D mutant increased the efficiency of NIK1-mediated RPL10 nuclear localization in co-transfected cells (Carvalho *et al.*, 2008; Santos *et al.*, 2009). Furthermore, NIK1 does not relocate a phosphorylation-deficient mutant of RPL10 to the nucleus (Carvalho *et al.*, 2008). Finally, mutations in the A-loop similarly affect the NIK1 capacity to mediate a phosphorylation-dependent nuclear relocalization of the RPL10 downstream component and to trigger an antiviral response (Carvalho *et al.*, 2008; Santos *et al.*, 2009). These data suggest that, although RPL10 is not a substrate for NIK1 protein, its nucleocytoplasmic shuttling is regulated by phosphorylation and is dependent on the kinase activity of NIK1, classifying RPL10 as a downstream effector of the NIK1-mediated signaling.

To gain further mechanistic insights into the role of NIK1 in antiviral immunity, the induced and repressed transcriptome by expressing the NIK1 phosphomimetic gain-of-function mutant T474D was assessed in Arabidopsis (Zorzatto *et al.*, 2015). NIK1 constitutive activation does not induce the expression of typical defense marker genes associated to gene silencing, salicylic acid, or PAMP-triggered immunity (PTI) pathways but rather it down-regulates translation-related genes, causing suppression of global *in vivo* translation and decreased loading of host mRNA in actively translating polysomes (PS) fractions. Likewise, induction of T474D expression through a

dexamethasone-inducible promoter also impairs global translation, which was correlated with a reduction of both PS and monosome (NPS) fractions, as well as of the RNA content associated with these fractions in the T474D lines. Ectopic expression of T474D controls begomovirus infection, causing symptomless infection, delayed course of infection and reduced accumulation of viral DNA in systemically infected leaves. Additionally, in infected T474D lines, the loading of coat protein viral mRNA in actively translating polysomes is reduced as compared to that of wild type infected lines, suggesting that the translation of viral transcripts is strongly impaired by NIK1-mediated signaling. Thus, begomovirus cannot sustain high levels of viral mRNA translation in the T474D-expressing lines, indicating that suppression of global protein synthesis may effectively protect plant cells against DNA viruses (Zorzatto *et al.*, 2015). Supporting this hypothesis, the T474D-overexpressing tomato transgenic lines are tolerant to the tomato-infecting begomoviruses ToYSV and *Tomato severe rugose virus* (ToSRV) (Brustolini *et al.*, 2015), which display highly divergent genomic sequences and hence are phylogenetically separated within the two major groups of begomoviruses found in Brazil (Albuquerque *et al.*, 2012). In addition, the gain-of-function mutant T474D from Arabidopsis functions similarly in tomato plants, as it causes a general down-regulation of translation machinery-related genes, affects translation in transgenic tomato lines and decreases viral mRNA association with the polysome fractions (Brustolini *et al.*, 2015). Therefore, the enhanced tolerance to tomato-infecting begomovirus displayed by the T474D-expressing lines is associated with the translational control branch of the NIK-mediated antiviral responses. These observations demonstrate the potential of a sustained NIK1-mediated defense pathway to confer broad-spectrum tolerance to begomoviruses in distinct plant species. Nevertheless, in the Arabidopsis homologous system, the level of translational inhibition by the constitutive activation of NIK1 causes stunted growth in transgenic lines grown under short-day conditions, whereas, in tomato, ectopic expression of the T474D mutant does not impact development under greenhouse conditions (Brustolini *et al.*, 2015; Zorzatto *et al.*, 2015). As a possible explanation for this phenotype, tomato plants may not need maximal translational capacity for optimal growth under greenhouse conditions; thereby, the level of translational inhibition mediated by NIK1 activation does not reach a threshold that would impact growth. Additionally or alternatively, the T474D-mediated translational suppression provokes a constant perception of stress in the transgenic lines, which, in turn, promotes acclimation to maintain normal growth under greenhouse conditions. Therefore, the intrinsic capacity of agronomically relevant crops to withstand the deleterious effect from the suppression of global translation is a relevant agronomic trait to be considered for engineering the NIK1-mediated resistance against begomoviruses in crops.

Recent progress towards directly connecting the NIK1-mediated signaling pathway with the downregulation of translational-machinery-related genes includes the isolation of a transcription factor harboring a MYB domain, named L10-INTERACTING MYB DOMAIN-CONTAINING PROTEIN (LIMYB), which interacts with RPL10 in the nucleus of plant cells (Zorzatto *et al.*, 2015). The interaction between LIMYB and RPL10 results in the formation of a transcriptional repressor complex that specifically suppresses the expression of ribosomal protein (RP) genes through the binding of LIMYB on RP gene promoters. This RP down-regulation leads to protein synthesis inhibition and enhanced tolerance to the begomovirus CaLCuV. T474D also down-regulates the expression of the same sub-set of LIMYB-regulated RP genes but requires the LIMYB function to repress RP gene expression. In addition, the loss of LIMYB function releases the repression of translation-related genes and increases susceptibility to CaLCuV infection (Zorzatto *et al.*, 2015). Collectively, these results provide both genetic and biochemical evidence that the LIMYB gene functions as a downstream component of the NIK1-mediated signaling pathway linking NIK1 activation to global translation suppression and tolerance to begomiviruses.

Despite the advances in the elucidation of NIK-mediated antiviral signaling pathway, there is a complete lack of information on the critical early event that triggers the NIK1 signaling and transduction, which culminates with the suppression of host global translation as an antiviral response. Recently, a comparison between the transcriptomes induced by begomovirus infection and by expression of the gain-of-function T474D mutant revealed that begomovirus infection is the activating stimulus of NIK1-mediated defense, although the molecular basis for this elicitation is still unknown (Machado *et al.*, 2015; Zorzatto *et al.*, 2015). A mechanistic model for a NIK1-mediated defense signaling pathway and its interaction with the begomovirus NSP is illustrated in Figure 3. Upon begomovirus infection, the extracellular domain of NIK undergoes oligomerization, allowing the intracellular kinase domains to transphosphorylate on a key threonine residue at position 474 (T474) and to activate one another (Santos *et al.*, 2009). Alternatively or additionally, NIK1 may serve as a co-receptor for a defense-signaling cascade and interacts with an unidentified ligand-dependent LRR-RLK receptor in response to virus infection. The phosphorylation-dependent activation of NIK leads to the phosphorylation of RPL10 and the phosphorylated RPL10 is translocated to the nucleus, where it interacts with LIMYB to fully down-regulate translation machinery-related genes, leading to host global translation suppression that affects the translation of the begomovirus mRNAs (Carvalho *et al.*, 2008; Zorzatto *et al.*, 2015). Thus, this down-regulation

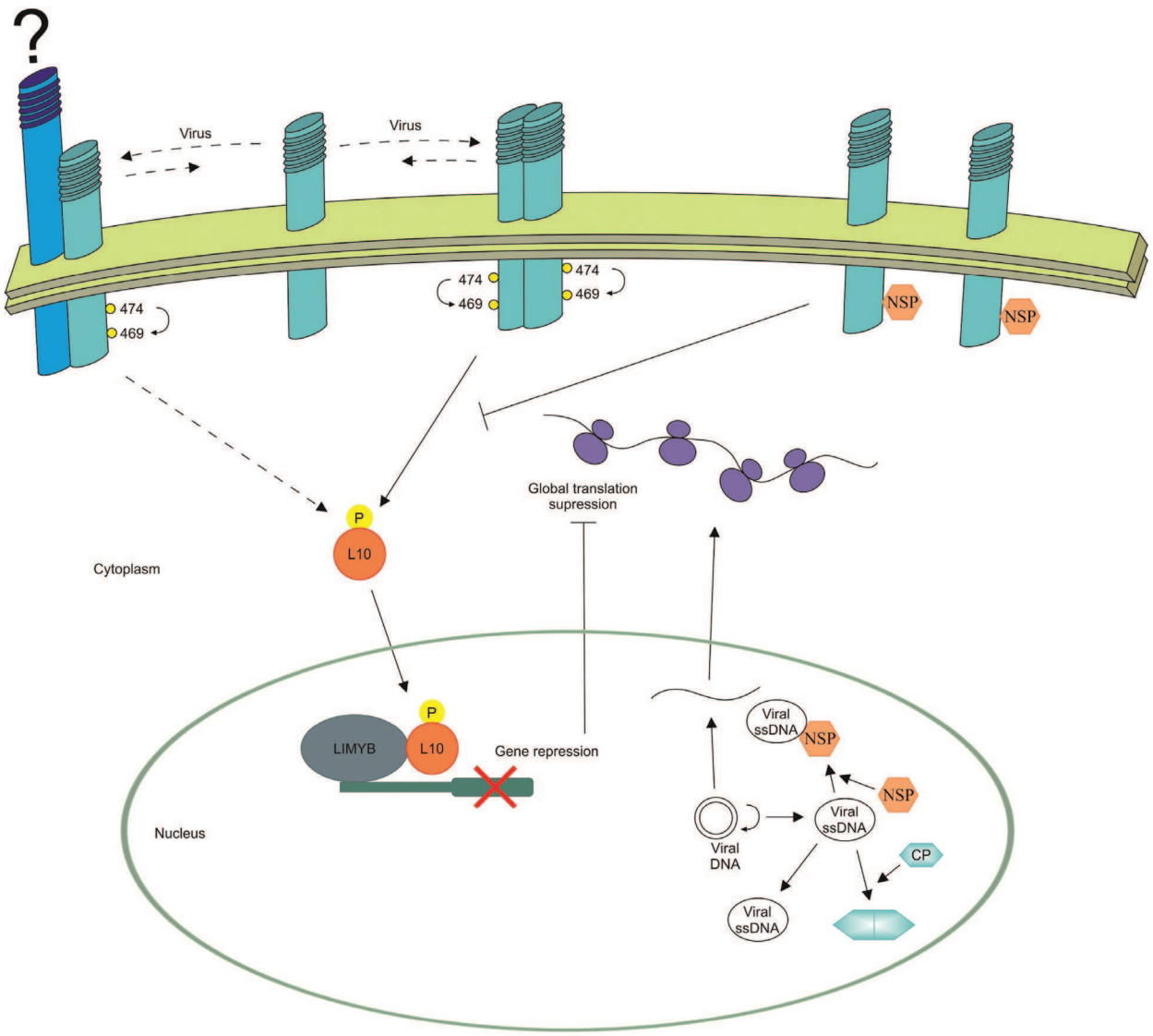

Figure 3 - Mechanistic overview of the antiviral defense pathway mediated by NIK1. Upon virus infection, NIK1 forms a homodimer and is activated through transphosphorylation of its kinase domain at the Thr-474 residue. Alternatively, NIK1 binds to an unknown ligand-binding LRR-RLK in a stimulus-dependent manner. The activation of NIK1 triggers the phosphorylation of RPL10 that, in turn, is transported to the nucleus. In the nuclear compartment, RPL10 interacts with LIMYB, which binds to the promoter of ribosomal protein (RP) genes to repress their transcription. As a consequence, a suppression of host global protein synthesis is observed, which also impairs translation of viral mRNA. As a defense countermeasure, NSP of begomoviruses binds and inhibits the NIK1 kinase activity, which impairs the RPL10 phosphorylation. Thus, RPL10 is retained in the cytosol, enhancing begomovirus infection. As begomoviruses have single-stranded circular DNA genomes, they replicate in the nucleus of the infected cells by double-stranded DNA intermediaries, which are also template for transcription of viral mRNAs. NSP binds to nascent viral DNA and facilitates the traffic of viral DNA from the nucleus to the cytoplasm by an unclear mechanism.

of cytosolic translation underlies at least partially the molecular mechanisms involved in the NIK1-mediated antiviral defense, which can be suppressed by binding of NSP to the NIK1 kinase domain.

Conclusions

Due to the agronomic importance of plant virus as pathogens, the development of antiviral strategies aiming crop protection has been continually on focus. In this context, the identification and characterization of host factors targeted during infection constitute one of the most important goals of the virology research. Due to their limited viral genome-encoded functions, the viruses have developed diverse strategies to hijack the host translation apparatus to quickly and efficiently produce viral proteins. Thus, translation repression has emerged as a plant antiviral defense strategy to impair the translation of viral proteins and could contribute as targets for the development of resistance strategy for virus control. In fact, plant RNA viruses interact tightly with the host protein synthesis machinery such that host translation initiation factor-encoding genes can function as recessive resistance genes. Furthermore, the trans-

lational repression activity of the effector AGO has been recently demonstrated to play a role in the antiviral RNA silencing mechanism. Finally, as a new paradigm in plant antiviral immunity, the activation of the immune receptor NIK1-mediated suppression of translation has been demonstrated to be effective in controlling begomovirus infections. These examples substantiate the notion that impairing viral mRNA translation (specifically or globally) constitutes a promising strategy for plant protection against viruses.

Acknowledgments

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (grants 573600/2008-2 and 447578/2014-6 to EPBF) and the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (grants CBB-APQ-03175-13 and CBB-APQ-01491-14 to EPBF). IPC was supported by a CAPES graduate fellowship and JPBM is a recipient of a CAPES postdoctoral fellowship.

References

Aitken CE and Lorsch JR (2012) A mechanistic overview of translation initiation in eukaryotes. Nat Struct Mol Biol 19:568-576.

Albar L, Bangratz-Reyser M, Hébrard E, Ndjiondjop MN, Jones M and Ghesquière A (2006) Mutations in the eIF(iso)4G translation initiation factor confer high resistance of rice to Rice yellow mottle virus. Plant J 47:417-426.

Albuquerque LC, Varsani A, Fernandes FR, Pinheiro B, Martin DP, Ferreira PTO, Lemos TO and Inoue-Nagata AK (2012) Further characterization of tomato-infecting begomoviruses in Brazil. Arch Virol 157:747-752.

Andrade M, Abe Y, Nakahara KS and Uyeda I (2009) The cyv-2 resistance to Clover yellow vein virus in pea is controlled by the eukaryotic initiation factor 4E. J Gen Plant Pathol 75:241-249.

Aukerman MJ and Sakai H (2003) Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. Plant Cell 15:2730-2741.

Azevedo J, Garcia D, Pontier D, Ohnesorge S, Yu A, Garcia S, Braun L, Bergdoll M, Hakimi MA, Lagrange T, *et al.* (2010) Argonaute quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. Genes Dev 24:904-915.

Baumberger N and Baulcombe DC (2005) Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. Proc Natl Acad Sci U S A 102:11928-11933.

Baumberger N, Tsai CH, Lie M, Havecker E and Baulcombe DC (2007) The Polerovirus silencing suppressor P0 targets ARGONAUTE proteins for degradation. Curr Biol 17:1609-1614.

Beauchemin C and Laliberte JF (2007) The poly(A) binding protein is internalized in virus-induced vesicles or redistributed to the nucleolus during Turnip mosaic virus infection. J Virol 81:10905-10913.

Bellon S, Fitzgibbon MJ, Fox T, Hsiao H-M and Wilson KP (1999) The structure of phosphorylated P38g is monomeric and reveals a conserved activation-loop conformation. Structure 7:1057-1065.

Bhattacharjee S, Zamora A, Azhar MT, Sacco MA, Lambert LH and Moffett P (2009) Virus resistance induced by NB-LRR proteins involves Argonaute4-dependent translational control. Plant J 58:940-951.

Biondi RM, Komander D, Thomas CC, Lizcano JM, Deak M, Alessi DR and van Aalten DM (2002) High resolution crystal structure of the human PDK1 catalytic domain defines the regulatory phosphopeptide docking site. EMBO J 21:4219-4228.

Bologna NG and Voinnet O (2014) The diversity, biogenesis, and activities of endogenous silencing small RNAs in Arabidopsis. Annu Rev Plant Biol 65:473-503.

Bortolamiol D, Pazhouhandeh M, Marrocco K, Genschik P and Ziegler-Graff V (2007) The Polerovirus F box protein P0 targets ARGONAUTE1 to suppress RNA silencing. Curr Biol 17:1615-1621.

Brodersen P, Sakvarelidze-Achard L, Bruun-Rasmussen M, Dunoyer P, Yamamoto YY, Sieburth L and Voinnet O (2008) Widespread translational inhibition by plant miRNAs and siRNAs. Science 320:1185-1190.

Brustolini OJB, Machado JPB, Condori-Apfata JA, Coco D, Deguchi M, Loriato VA, Pereira W, Alfenas-Zerbini P, Zerbini FM, Inoue-Nagata AK, *et al.* (2015) Sustained NIK-mediated antiviral signalling confers broad-spectrum tolerance to begomoviruses in cultivated plants. Plant Biotechnol J 13:1300-1311.

Burgyán J and Havelda Z (2011) Viral suppressors of RNA silencing. Trends Plant Sci 16:265-272.

Carbonell A and Carrington JC (2015) Antiviral roles of plant ARGONAUTES. Curr Opin Plant Biol 27:111-117.

Carvalho CM, Santos AA, Pires SR, Rocha SR, Saraiva DI, Machado JP, Mattos EC, Fietto LG and Fontes EP (2008) Regulated nuclear trafficking of rpL10A mediated by NIK1 represents a defense strategy of plant cells against viruses. PLoS Pathog 4:e1000247.

Chen X (2004) A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science 303:2022-2025.

Chiu MH, Chen IH, Baulcombe DC and Tsai CH (2010) The silencing suppressor P25 of Potato virus X interacts with Argonaute1 and mediates its degradation through the proteasome pathway. Mol Plant Pathol 11:641-649.

Contreras-Paredes CA, Silva-Rosales L, Daròs JA, Alejandri-Ramírez ND and Dinkova TD (2013) The absence of eukaryotic initiation factor eIF(iso)4E affects the systemic spread of a tobacco etch virus isolate in *Arabidopsis thaliana*. Mol Plant-Microbe Interact J 26:461-470.

Csorba T, Lozsa R, Hutvagner G and Burgyan J (2010) Polerovirus protein P0 prevents the assembly of small RNA-containing RISC complexes and leads to degradation of ARGONAUTE1. Plant J 62:463-472.

Csorba T, Kontra L and Burgyan J (2015) Viral silencing suppressors: Tools forged to fine-tune host-pathogen coexistence. Virology 479-480:85-103.

De Ronde D, Butterbach P and Kormelink R (2014) Dominant resistance against plant viruses. Front Plant Sci 5:307.

Derrien B, Baumberger N, Schepetilnikov M, Viotti C, De Cillia J, Ziegler-Graff V, Isono E, Schumacher K and Genschik P (2012) Degradation of the antiviral component ARGONAUTE1 by the autophagy pathway. Proc Natl Acad Sci U S A 109:15942-15946.

Dievart A and Clark SE (2004) LRR-containing receptors regulating plant development and defense. Development 131:251-261.

Ding SW (2010) RNA-based antiviral immunity. Nat Rev Immunol 10:632-644.

Eskelin K, Hafren A, Rantalainen KI and Makinen K (2011) Potyviral VPg enhances viral RNA Translation and inhibits reporter mRNA translation in planta. J Virol 85:9210-9221.

Fang X and Qi Y (2016) RNAi in Plants: An Argonaute-centered view. Plant Cell 28:272-285.

Fátyol K, Ludman M and Burgyán J (2016) Functional dissection of a plant Argonaute. Nucleic Acids Res 44:1384-1397.

Fontes EPB, Santos AA, Luz DF, Waclawovsky AJ and Chory J (2004) The geminivirus nuclear shuttle protein is a virulence factor that suppresses transmembrane receptor kinase activity. Genes Dev 18:2545-2556.

Fukaya T and Tomari Y (2012) MicroRNAs mediate gene silencing via multiple different pathways in Drosophila. Mol Cell 48:825-836.

Fusaro AF, Correa RL, Nakasugi K, Jackson C, Kawchuk L, Vaslin MF and Waterhouse PM (2012) The Enamovirus P0 protein is a silencing suppressor which inhibits local and systemic RNA silencing through AGO1 degradation. Virology 426:178-187.

Galvez LC, Banerjee J, Pinar H and Mitra A (2014) Engineered plant virus resistance. Plant Sci 228:11-25.

Gandikota M, Birkenbihl RP, Höhmann S, Cardon GH, Saedler H and Huijser P (2007) The miRNA156/157 recognition element in the 3' UTR of the Arabidopsis SBP box gene SPL3 prevents early flowering by translational inhibition in seedlings. Plant J 49:683-693.

Gao Z, Eyers S, Thomas C, Ellis N and Maule A (2004a) Identification of markers tightly linked to sbm recessive genes for resistance to Pea seed-borne mosaic virus. Theor Appl Genet 109 488-494.

Gao Z, Johansen E, Eyers S, Thomas CL, Noel Ellis TH and Maule AJ (2004b) The potyvirus 9 recessive resistance gene, sbm1, identifies a novel role for translation initiation factor 10 eIF4E in cell-to-cell trafficking. Plant J 40:376-385.

Garcia-Ruiz H, Carbonell A, Hoyer JS, Fahlgren N, Gilbert KB, Takeda A, Giampetruzzi A, Garcia Ruiz MT, McGinn MG, Lowery N, *et al.* (2015) Roles and programming of Arabidopsis ARGONAUTE proteins during turnip mosaic virus infection. PLoS Pathog 11:e1004755.

Ghoshal B and Sanfaçon H (2014) Temperature-dependent symptom recovery in Nicotiana benthamiana plants infected with tomato ringspot virus is associated with reduced translation of viral RNA2 and requires ARGONAUTE 1. Virology 456-457:188-197.

Ghoshal B and Sanfaçon H (2015) Symptom recovery in virus-infected plants: Revisiting the role of RNA silencing mechanisms. Virology 479-480:167-179.

Giner A, Lakatos L, Garcia-Chapa M, Lopez-Moya JJ and Burgyán J (2010) Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs. PLoS Pathog 6:e1000996.

Goodfellow IG and Roberts LO (2008) Eukaryotic initiation factor 4E. Int J Biochem Cell Biol 40:2675-2680.

Gururani MA, Venkatesh J, Upadhyaya CP, Nookaraju A, Pandeyc SK and Park SW (2012) Plant disease resistance genes: Current status and future directions. Physiol Mol Plant Pathol 78:51-65.

Hafren A, Eskelin K and Mäkinen K (2013) Ribosomal protein P0 promotes Potato virus A infection and functions in viral translation together with VPg and eIF(iso)4E. J Virol 87:4302-4312.

Hanks SK, Quinn AM and Hunter T (1988) The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. Science 241:42-52.

Hanley-Bowdoin L, Bejarano ER, Robertson D and Mansoor S (2013) Geminiviruses: Masters at redirecting and reprogramming plant processes. Nat Rev Microbiol 11:777-788.

Hebrard E, Poulicard N, Gerard C, Traore O, Wu HC, Albar L, Fargette D, Bessin Y and Vignols F (2010) Direct interaction between the Rice yellow mottle virus (RYMV) VPg and the central domain of the rice eIF(iso)4G1 factor correlates with rice susceptibility and RYMV virulence. Mol Plant Microbe Interact 23:1506-1513.

Hinnebusch AG (2014) The scanning mechanism of eukaryotic translation initiation. Annu Rev Biochem 83:779-812.

Huang TS, Wei T, Laliberte JF and Wang A (2010) A host RNA helicase-like protein, AtRH8, interacts with the potyviral genome-linked protein, VPg, associates with the virus accumulation complex, and is essential for infection. Plant Physiol 152:255-266.

Hubbard SR (1997) Crystal structure of the active insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. EMBO J 16:5573-5581.

Huntzinger E and Izaurralde E (2011) Gene silencing by microRNAs: Contributions of translational repression and mRNA decay. Nat Rev Genet 12:99-110.

Hwang J, Oh CS and Kang BC (2013) Translation elongation factor 1B (eEF1B) is an essential host factor for Tobacco mosaic virus infection in plants. Virology 439:105-114.

Iwakawa HO and Tomari Y (2013) Molecular insights into microRNAmediated translational repression in plants. Mol Cell 52:591-601.

Jackson RJ, Hellen CU and Pestova TV (2010) The mechanism of eukaryotic translation initiation and principles of its regulation. Nat Rev Mol Cell Biol 11:113-127.

Jones-Rhoades MW, Bartel DP and Bartel B (2006) MicroRNAs and their regulatory roles in plants. Annu Rev Plant Biol 57:19-53.

Julio E, Cotucheau J, Decorps C, Volpatti R, Sentenac C, Candresse T and Dorlhac de Borne F (2015) A eukaryotic translation initiation factor 4E (eIF4E) is responsible for the "va" tobacco recessive resistance to potyviruses. Plant Mol Biol Rep 33:609-623.

Kamthan A, Chaudhuri A, Kamthan M and Datta A (2015) Small RNAs in plants: Recent development and application for crop improvement. Front Plant Sci 6:208.

Kang BC, Yeam I, Frantz JD, Murphy JD and Jahn MM (2005a) The pvr1 locus in Capsicum encodes a translation initiation factor eIF4E that interacts with Tobacco etch virus VPg. Plant J 42:392-405.

Kang BC, Yeam I and Jahn MM (2005b) Genetics of plant virus resistance. Annu Rev Phytopathol 43:581-621.

Karran RA and Sanfaçon H (2014) Tomato ringspot virus coat protein binds to ARGONAUTE 1 and suppresses the translation repression of a reporter gene. Mol Plant Microbe Interact 27:933-943.

Kasschau KD and Carrington JC (1998) A counterdefensive strategy of plant viruses: Suppression of posttranscriptional gene silencing. Cell 95:461-470.

Khan MA, Miyoshi H, Gallie DR and Goss DJ (2008) Potyvirus genome-linked protein, Vpg, directly affects wheat germ in vitro translation: Interactions with translation initiation factors eIF4F and eIFiso4F. J Biol Chem 283:1340-1349.

Kornev AP, Haste NH, Taylor SS and Eyck LFT (2006) Surface comparision of active and inactive protein kinases identifies a conserved activation mechanism. Proc Natl Acad Sci USA 103:17783-17788.

Krol J, Loedige I and Filipowicz W (2010) The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 11:597-610.

Lanet E, Delannoy E, Sormani R, Floris M, Brodersen P, Crété P, Voinnet O and Robaglia C (2009) Biochemical evidence for translational repression by Arabidopsis microRNAs. Plant Cell 21:1762-1768.

Lee JH, Muhsin M, Atienza GA, Kwak DY, Kim SM, De Leon TB, Angeles ER and Coloquio E (2010). Single nuclieotide polymorphisms in a gene for translation initiation factor (eIF4G) of rice (*Oryza sativa*) associated with resistance to rice tungro spherical virus. Mol Plant-Microbe Interact J 23:29-38.

Lellis AD, Kasschau KD, Whitham SA and Carrington JC (2002) Loss-of-susceptibility mutants of *Arabidopsis thaliana* reveal an essential role for eIF(iso)4E during potyvirus infection. Curr Biol 12:1046-1051.

Leonard S, Plante D, Wittmann S, Daigneault N, Fortin MG and Laliberté JF (2000) Complex formation between potyvirus VPg and translation eukaryotic initiation factor 4E correlates with virus infectivity. J Virol 74:7730-7737.

Li S, Liu L, Zhuang X, Yu Y, Liu X, Cui X, Ji L, Pan Z, Cao X, Mo B, *et al.* (2013) MicroRNAs inhibit the translation of target mRNAs on the endoplasmic reticulum in Arabidopsis. Cell 153:562-574.

Llave C, Xie Z, Kasschau KD and Carrington JC (2002) Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science 297:2053-2056.

Ma X, Nicole MC, Meteignier LV, Hong N, Wang G and Moffett P (2015) Different roles for RNA silencing and RNA processing components in virus recovery and virus-induced gene silencing in plants. J Exp Bot 66:919-932.

Machado JPB, Brustolini OJB, Mendes GC, Santos AA and Fontes EPB (2015) NIK1, a host factor specialized in antiviral defense or a novel general regulator of plant immunity? BioEssays 37:1236-1242.

Mallory A and Vaucheret H (2010) Form, function, and regulation of ARGONAUTE proteins. Plant Cell 22:3879-3889.

Mandadi KK and Scholthof K-BG (2013) Plant immune responses against viruses: How does a virus cause disease? Plant Cell 25:1489-1505.

Mariano AC, Andrade MO, Santos AA, Carolino SMB, Oliveira ML, Baracat-Pereira MC, Brommonshenkel SH and Fontes EP (2004) Identification of a novel receptor-like protein kinase that interacts with a geminivirus nuclear shuttle protein. Virology 318:24-31.

Martinez de Alba AE, Elvira-Matelot E and Vaucheret H (2013) Gene silencing in plants: A diversity of pathways. Biochim Biophys Acta 1829:1300-1308.

Matsuda D and Dreher TW (2004) The tRNA-like structure of Turnip yellow mosaic virus RNA is a 3'-translational enhancer. Virology 321:36-46.

Michon T, Estevez Y, Walter J, German-Retana S and Le Gall O (2006) The potyviral virus genome-linked protein VPg forms a ternary complex with the eukaryotic initiation factors eIF4E and eIF4G and reduces eIF4E affinity for a mRNA cap analogue. FEBS J 273:1312-1322.

Nicaise V (2014) Crop immunity against viruses: Outcomes and future challenges. Front Plant Sci 5:660.

Nicaise V, German-Retana S, Sanjuan R, Dubrana MP, Mazier M, Maisonneuve B, Candresse T, Caranta C and LeGall O (2003) The eukaryotic translation initiation factor 4E controls lettuce susceptibility to the Potyvirus Lettuce mosaic virus. Plant Physiol 132:1272-1282.

Nicaise V, Gallois JL, Chafiai F, Allen LM, Schurdi-Levraud V, Browning KS, Candresse T, Caranta C, Le Gall O and German-Retana S (2007) Coordinated and selective recruitment of eIF4E and eIF4G factors for potyvirus infection in Arabidopsis thaliana. FEBS Lett 581:1041-1046.

Nieto C, Morales M, Orjeda G, Clepet C, Monfort A, Sturbois B, Puigdomènech P, Pitrat M, Caboche M, Dogimont C, *et al.* (2006) An eIF4E allele confers resistance to an uncapped and non-polyadenylated RNA virus in melon. Plant J 48:452-462.

Osman TA and Buck KW (1997) The Tobacco mosaic virus RNA polymerase complex contains a plant protein related to the RNA-binding subunit of yeast eIF-3. J Virol 71:6075-6082.

Patrick RM and Browning KS (2012) The eIF4F and eIFiso4F complexes of plants: An evolutionary perspective. Comp Funct Genomics 2012:287814.

Pfaff J and Meister G (2013) Argonaute and GW182 proteins: An effective alliance in gene silencing. Biochem Soc Trans 41:855-860.

Plante D, Viel C, Leonard S, Tampo H, Laliberté b J-F and Fortin MG (2004) Turnip mosaic virus VPg does not disrupt the translation initiation complex but interfere with cap binding. Physiol Mol Plant Pathol 64:219-226.

Pumplin N and Voinnet O (2013) RNA silencing suppression by plant pathogens: Defence, counter-defence and counter-counter-defence. Nat Rev Microbiol 11:745-760.

Rajamäki ML and Valkonen JPT (2009) Control of nuclear and nucleolar localization of nuclear inclusion protein a of picorna-like Potato virus A in Nicotiana species. Plant Cell 21:2485-2502.

Ritzenthaler C (2005) Resistance to plant viruses: Old issue, news answers? Curr Opin Biotechnol 16:118-122.

Robaglia C and Caranta C (2006) Translation initiation factors: A weak link in plant RNA virus infection. Trends Plant Sci 11:40-45.

Rocha CS, Santos AA, Machado JPB and Fontes EPB (2008) The ribosomal protein L10/QM-like protein is a component of the NIK-mediated antiviral signaling. Virology 380:165-169.

Ruffel S, Dussault MH, Palloix A, Moury B, Bendahmane A, Robaglia C and Caranta C (2002) A natural recessive resis-

tance gene against Potato virus Y in pepper corresponds to the eukaryotic initiation factor 4E (eIF4E). Plant J 32:1067-1075.

Ruffel S, Gallois JL, Lesage ML and Caranta C (2005) The recessive potyvirus resistance gene pot-1 is the tomato orthologue of the pepper pvr2-eIF4E gene. Mol Genet Genomics 274:346-353.

Ruffel S, Gallois JL, Moury B, Robaglia C, Palloix A and Caranta C (2006) Simultaneous mutations in translation initiation factors eIF4E and eIF(iso)4E are required to prevent pepper veinal mottle virus infection of pepper. J Gen Virol 87:2089-2098.

Sakamoto T, Deguchi M, Brustolini OJB, Santos AA, Silva FF and Fontes EP (2012) The tomato RLK superfamily: Phylogeny and functional predictions about the role of the LRRII-RLK subfamily in antiviral defense. BMC Plant Biol 12:229.

Sanfaçon H (2015) Plant translation factors and virus resistance. Viruses 7:3392-3419.

Santos AA, Carvalho CM, Florentino LH, Ramos HJO and Fontes EP (2009) Conserved threonine residues within the A-loop of the receptor NIK differentially regulate the kinase function required for antiviral signaling. PLoS One 4:e5781.

Schaad MC, Anderberg RJ and Carrington JC (2000) Strain-specific interaction of the tobacco etch virus NIa protein with the translation initiation factor eIF4E in the yeast two-hybrid system. Virology 273:300-306.

Shiu S-H and Bleecker AB (2001) Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. Proc Natl Acad Sci U S A 98:10763-10768.

Stein N, Perovic D, Kumlehn J, Pellio B, Stracke S, Streng S, Ordon F and Graner A (2005) The eukaryotic translation initiation factor 4E confers multiallelic recessive Bymovirus resistance in *Hordeum vulgare* (L.). Plant J 42:912-922.

Szabo EZ, Manczinger M, Goblos A, Kemeny L and Lakatos L (2012) Switching on RNA silencing suppressor activity by restoring argonaute binding to a viral protein. J Virol 86 8324-8327.

Szittya G and Burgyan J (2013) RNA interference-mediated intrinsic antiviral immunity in plants. Curr Top Microbiol Immunol 371:153-181.

Tang G, Reinhart BJ, Bartel DP and Zamore PD (2003) A biochemical framework for RNA silencing in plants. Genes Dev 17:49-63.

Thivierge K, Cotton S, Dufresne PJ, Mathieu I, Beauchemin C, Ide C, Fortin MG and Laliberté JF (2008) Eukaryotic elongation factor 1A interacts with Turnip mosaic virus RNA-dependent RNA polymerase and VPg-Pro in virus-induced vesicles. Virology 377:216-225.

Truniger V and Aranda MA (2009) Recessive resistance to plant viruses. Adv Virus Res 75:119-159.

Vargason JM, Szittya G, Burgyan J and Tanaka Hall TM (2003) Size selective recognition of siRNA by an RNA silencing suppressor. Cell 115:799-811.

Walsh D and Mohr I (2011) Viral subversion of the host protein synthesis machinery. Nat RevMicrobiol 9:860-875.

Wang A (2015) Dissecting the molecular network of virus-plant interactions: The complex roles of host factors. Annu Rev Phytopathol 53:45-66.

Wang A and Krishnaswamy S (2012) Eukaryotic translation initiation factor 4E-mediated recessive resistance to plant viruses and its utility in crop improvement. Mol Plant Pathol 13:795-803.

Wang H-LV and Chekanova JA (2016) Small RNAs: Essential regulators of gene expression and defenses against environmental stresses in plants. Wiley Interdiscip Rev RNA 7:356-381.

Wilczynska A and Bushell M (2015) The complexity of miRNA-mediated repression. Cell Death Differ 22:22-33.

Wittmann S, Chatel H, Fortin MG and Laliberte JF (1997) Interaction of the viral protein genome linked of Turnip mosaic potyvirus with the translational eukaryotic initiation factor (iso)4E of *Arabidopsis thaliana* using the yeast two-hybrid system. Virology 234:84-92.

Yamaji Y, Kobayashi T, Hamada K, Sakurai K, Yoshii A, Suzuki M, Namba S and Hibi T (2006) *In vivo* interaction between Tobacco mosaic virus RNA-dependent RNA polymerase and host translation elongation factor 1A. Virology 347:100-108.

Yang J, Cron P, Thompson V, Good VM, Hess D, Hemmings BA and Barford D (2002) Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation. Mol Cell 9:1227-1240.

Yang L, Wu G and Poethig RS (2012) Mutations in the GW-repeat protein SUO reveal a developmental function for microRNA-mediated translational repression in Arabidopsis. Proc Natl Acad Sci U S A 109:315-320.

Ye K, Malinina L and Patel DJ (2003) Recognition of small interfering RNA by a viral suppressor of RNA silencing. Nature 426:874-878.

Yoshii M, Nishikiori M, Tomita K, Yoshioka N, Kozuka R, Naito S and Ishikawa M (2004) The Arabidopsis Cucumovirus multiplication 1 and 2 loci encode translation initiation factors 4E and 4G. J Virol 78:6102-6111.

Zhang C, Wu Z, Li Y and Wu J (2015) Biogenesis, function, and applications of virus-derived small RNAs in plants. Front Microbiol 6:1237.

Zhang X, Yuan YR, Pei Y, Lin SS, Tuschl T, Patel DJ and Chua NH (2006) Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute1 cleavage activity to counter plant defense. Genes Dev 20:3255-3268.

Zhang X, Zhang X, Singh J, Li D and Qu F (2012) Temperature-dependent survival of Turnip crinkle virus-infected arabidopsis plants relies on an RNA silencing-based defense that requires dcl2, AGO2, and HEN1. J Virol 86:6847-6854.

Zorzatto C, Machado JPB, Lopes KVG, Nascimento KJT, Pereira WA, Brustolini OJ, Reis PA, Calil IP, Deguchi M, Sachetto-Martins G, *et al.* (2015) NIK1-mediated translation suppression functions as a plant antiviral immunity mechanism. Nature 520:679-682.

Associate Editor: Marcio de Castro Silva Filho