Brassica yellows virus P0 protein impairs the antiviral activity of NbRAF2 in *Nicotiana benthamiana*

Qian Sun1, Yuan-Yuan Li1, Ying Wang1, Hang-Hai Zhao1, Tian-Yu Zhao1, Zong-Ying Zhang1, Da-Wei Li2, Jia-Lin Yu2, Xian-Bing Wang2, Yong-Liang Zhang2 and Cheng-Gui Han1,*

1 State Key Laboratory for Agro-biotechnology and Ministry of Agriculture Key Laboratory of Plant Pathology, China Agricultural University, Beijing 100193, P. R. China
2 State Key Laboratory of Agro-Biotechnology and Ministry of Agriculture Key Laboratory of Soil Microbiology, College of Biological Sciences, China Agricultural University, Beijing 100193, P. R., China

* Correspondence: hanchenggui@cau.edu.cn

Received 7 December 2017; Editorial decision 22 March 2018; Accepted 24 March 2018

Editor: Chris Hawes, Oxford Brookes University, UK

Abstract

In interactions between poleroviruses and their hosts, few cellular proteins have been identified that directly interact with the multifunctional virus P0 protein. To help explore the functions of P0, we identified a Brassica yellows virus genotype A (BrYV-A) P0BrA-interacting protein from *Nicotiana benthamiana*, Rubisco assembly factor 2 (NbRAF2), which localizes in the nucleus, cell periphery, chloroplasts, and stromules. We found that its C-terminal domain (amino acids 183–211) is required for self-interaction. A split ubiquitin membrane-bound yeast two-hybrid system and co-immunoprecipitation assays showed that NbRAF2 interacted with P0BrA, and co-localized in the nucleus and at the cell periphery. Interestingly, the nuclear pool of NbRAF2 decreased in the presence of P0BrA and during BrYV-A infection, and the P0BrA-mediated reduction of nuclear NbRAF2 required dual localization of NbRAF2 in the chloroplasts and nucleus. *Tobacco rattle virus*-based virus-induced gene silencing of NbRAF2 promoted BrYV-A infection in *N. benthamiana*, and the overexpression of nuclear NbRAF2 inhibited BrYV-A accumulation. *Potato leafroll virus* P0PL also interacted with NbRAF2 and decreased its nuclear accumulation, indicating that NbRAF2 may be a common target of poleroviruses. These results suggest that nuclear NbRAF2 possesses antiviral activity against BrYV-A infection, and that BrYV-A P0BrA interacts with NbRAF2 and alters its localization pattern to facilitate virus infection.

Keywords: Brassica yellows virus, NbRAF2, *Nicotiana benthamiana*, nuclear localization, P0, stromules.

Introduction

Numerous studies have demonstrated that chloroplasts are a common target of many plant viruses. Chloroplasts and their components are involved in viral movement, replication, and symptom development, and also participate in plant defense against viruses (Zhao et al., 2016; Bhattacharyya and Chakraborty, 2018). Several chloroplast proteins have been shown to play negative roles in viral pathogenesis. The coat protein of *Alfalfa mosaic virus* interacts with the photosystem-II oxygen-evolving complex protein PsbP, and overexpression of PsbP markedly reduces virus accumulation (Balasubramaniam et al., 2014). The cylindrical inclusion protein from *Plum pox virus* (PPV) binds to the photosystem-I PSI-K protein, which is the product of *psaK*. PPV infection down-regulates the expression of *psaK* mRNA in inoculated leaves, and the silencing of *psaK* leads to a greater accumulation of PPV (Jiménez et al., 2006). Pathogens attempt to...
intercept chloroplast proteins by interacting and sequestering them in the cytosol before they are imported into the chloroplasts or by affecting their normal activities (Jin et al., 2007; Shi et al., 2007; Balasubramaniam et al., 2014; Kong et al., 2014).

In mammals, the single pterin-4a-carbinolamine dehydratase/dimerization co-factor of hepatocyte nuclear factor 1 (PCD/DCoH) acts as a metabolic enzyme, and has PCD activity in the mitochondria and also acts as a DCoH in the nucleus (Suck and Finer, 1996). The activities of PCD and DCoH are independent (Rhee et al., 1997). In plants, there are two copies of PCD/DCoH homologs (Naponelli et al., 2008). Type I is localized to the mitochondria and has PCD activity, whereas type 2, without PCD activity, is directed to the chloroplast (Naponelli et al., 2008). The Arabidopsis protein Rubisco assembly factor 2 (AtRAF2) (Fristedt et al., 2018), also named SDIR1P1 (Zhang et al., 2015a) or ATP1 (Oh et al., 2017), is a type 2 protein of the PCD/DCoH family (Naponelli et al., 2008). AtRAF2 is nuclear-encoded and localized to the chloroplast (Naponelli et al., 2008; Zybaílov et al., 2008). It has been suggested that AtSDIRIP1/AtRAF2 is localized not only to the chloroplast but also to the cell periphery and nucleus (Zhang et al., 2015a). The chloroplast/nuclear protein AtSDIRIP1/AtRAF2 interacts with the E3 ligase SDIR1 in the cytosol, and is subsequently degraded by it. AtSDIRIP1/AtRAF2 selectively regulates the expression of the abscisic acid (ABA)-responsive transcription factor gene ABA-INSENSITIVE5 to regulate ABA-mediated seed germination and salt-stress responses (Zhang et al., 2015a). The cytosolic RING-type E3 ligase AtAIRP2 also targets AtATP1/AtRAF2 for degradation, and AtAIRP2 and AtSDIR1 play a combinatorial role in ABA- and salt-stress responses in Arabidopsis (Oh et al., 2017). In addition, Zea mays RAF2 (ZmRAF2) shares a high amino acid sequence identity with AtRAF2 (Naponelli et al., 2008), and both proteins are involved in Rubisco assembly (Feiz et al., 2014; Fristedt et al., 2018). However, in plants, the function of RAF2 in the nucleus remains unknown.

Poleroviruses, belonging to the family Luteoviridae, infect many crops of economic importance and cause serious yield losses (Taliansky et al., 2003; Stevens et al., 2005). They cause yellowing symptoms in a wide range of hosts (Scaglusi and Lockhart, 2000; Peter et al., 2009; Xiang et al., 2011; Chen et al., 2016). Viruses of this family have a positive-sense RNA genome of 5000–6000 nt, from which the P0 protein encoded by ORF0 has been shown to suppress RNA silencing (Pfeffer et al., 2002; Taliansky et al., 2003; Stevens et al., 2005; Pazhouhandeh et al., 2006; Mangwende et al., 2009; Csorba et al., 2010; Han et al., 2010; Kozlowska-Makul ska et al., 2010; Delfosse et al., 2014; Zhuo et al., 2014; Chen et al., 2016). The P0 proteins can interact with S-phase kinase-associated protein 1 (SKP1), a member of the SKP1–Cullin 1–F-box E3 ubiquitin ligase complex, and trigger the ubiquitylation and degradation of Argonaute1 (AGO1) in plants (Pazhouhandeh et al., 2006; Bortolomioli et al., 2007; Csorba et al., 2010; Fusaro et al., 2012). P0 also physically interacts with AGO1 in the nucleus, where ASK1/2 and AtCUL1 localize, which supports the hypothesis that AGO1 could be a direct target of P0 (Bortolomioli et al., 2007). However, AGO1 degradation by P0 is blocked by the inhibition of autophagy (Derrien et al., 2012) but not proteasomes (Baumberger et al., 2007). In addition, the P0 proteins induce cell death within the infiltration patch in Nicotiana species (Mangwende et al., 2009; Csorba et al., 2010; Fusaro et al., 2012; Wang et al., 2015). Turnip yellow virus P0P1, Potato leafroll virus (PLRV) P0PL, and Cucurbit aphid-borne yellow virus P0C9 elicit a hypersensitive response in the N. glutinosa accession TW59 (Wang et al., 2015). A genetic analysis showed that P0C9 is recognized by a resistance gene, designated Resistance to Poleroviruses 1, and functions in an E3 ubiquitin ligase complex as a potential trigger of Resistance to Poleroviruses 1-mediated effectector-triggered immunity (Wang et al., 2015). However, few host proteins that directly interact with the P0 protein have been identified (Pazhouhandeh et al., 2006; Bortolomioli et al., 2007).

Brassica yellows virus (BrYV), a newly identified polerovirus, infects crucifer crops in China and causes yellowing or leaf-roll symptoms (Xiang et al., 2011). BrYV has three genotypes, BrYV-A, B, and C (Xiang et al., 2011; Zhang et al., 2014), and the full-length infectious cDNA clones of these three genotypes have been developed successfully (Zhang et al., 2015b). The BrYV P0 is a strong viral suppressor of RNA silencing and interacts with the SKP1 from N. benthamiana (Xiang and Han, 2011). In the present study, we successfully obtained a novel P0BrA-interacting protein, NbRAF2, which localized to the nucleus, cell periphery, chloroplasts, and stomates. We demonstrated that P0BrA decreased the nuclear accumulation of NbRA2. The accumulation of BrYV-A increased when NbRAF2 was silenced using Tobacco rattle virus (TRV)-based virus induced gene silencing (VIGS) but decreased when nuclear NbRAF2 was overexpressed. PLRV P0Pl also interacted with NbRAF2 and decreased its nuclear pool, indicating that nuclear NbRAF2 may be a common target of polerovirus P0s.

Materials and methods

Plant material and growth conditions

Wild-type Nicotiana benthamiana and transgenic lines with the ferredoxin NADP(H) oxidoreductase transit peptide fused to enhanced green fluorescent protein (FNR–EGFP) (Schattat et al., 2011) were grown at 24°C with a 16-h light/8-h dark cycle.

Plasmid constructs

All of the primers used in this study are listed in Supplementary Table S1 at JXB online.

The vectors pGD and pGDG (Goodin et al., 2002) were used for transient expression. The P38 protein encoded by Turnip crinkle virus was cloned into pGD for transient expression. P0BrA was cloned into pGD–3Flag, a modified version of vector pGD that has a C-terminal fused 3×Flag tag. For co-immunoprecipitation (Co-IP) and confocal microscopy, pGD–3G-mCherry was constructed. A DNA fragment of GGG-mCherry was amplified and cloned into the vector pGD to produce pGD–3G-mCherry. Full-length AtAIRP2, NbRAF2, and their mutants were independently cloned into the vector pGDGm [a modified version of pGD that allows the production of a C-terminal green fluorescent protein (GFP)-fused protein] and pGD–3G-mCherry. NbSKP1 was cloned into the vector...
Agrobacterium-mediated transient expression in N. benthamiana

Plasmids were transformed into the Agrobacterium tumefaciens strain EHA105 or C58CI using the freeze-thaw method (Holsters et al., 1978). The recombinant EHA105 or C58CI was grown overnight, resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES, and 100 μM acetosyringone), and incubated at room temperature for at least 3 h before infiltration. The A. tumefaciens cultures were infiltrated into N. benthamiana leaves and the infiltrated leaves were detached for the corresponding assays.

Yeast two-hybrid (Y2H) screen and interaction assays

For the Y2H assay, the Clontech Matchmaker GAL4 Two-Hybrid System 3 (Clontech, Mountain View, CA, USA) was used. Protein-interacting screens were performed with the Matchmaker GAL4 two-hybrid system according to the manufacturer’s protocol. The full-length of P0BrA was cloned into pGBK T7 containing a binding domain (BD) to generate BD-P0BrA and then transformed into the yeast host strain Y187. The Arabidopsis cDNA library was used to screen BD-P0BrA-binding proteins. The full-length AtRAF2 was cloned into pGAD T7 containing an activating domain (AD) to generate AD-AFRA2 and then transformed into the yeast host strain AH109. Co-transformants were plated on synthetic dropout media lacking Trp and Leu (SD/−WL) and SD/−AHLW plates for 3–5 d.

Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer’s protocol. A 4-μg total RNA was treated with RNase-free DNase I (TakaRa). First-strand cDNA was synthesized using 2 μg of treated RNA, oligo d(T) primer, or gene-specific primer and M-MLV Reverse Transcriptase (Promega), as instructed by the protocol. The mRNAs of EF1A, NbRAF2, and BrYV-A RNA were determined using specific primers (see Supplementary Table S1).

Fluorescence microscopy

Agro-infiltrated leaf tissue from N. benthamiana was detached 2 d post infiltration (dpi), and confocal laser scanning microscopy was performed using a Leica SPS laser-scanning microscope. GFP, mCherry, and chloroplast fluorescence were obtained using laser excitation at 488, 552, and 638 nm, respectively. For bimolecular fluorescence complementation (BiFC) assays, NbRAF2 and AtRAF2 were respectively cloned into pSPYNE–35S and pSPYCE–35S (provided by J. Kudla, Universität Münster, Germany). Yellow fluorescent protein (YFP) fluorescence was obtained using 488-nm laser excitation. To determine its distribution, the nuclear-localized NbRAF2 was quantified from Z stacks and expressed as the total number of nuclear-localized NbRAF2 particles per total number of nuclei.

Nucleocytoplasmatic fractionation assay

Nucleocytoplasmatic fractionation was performed using a Celllytic™ PN Isolation/Extraction kit following the manufacturer’s instructions (Sigma-Aldrich). Total, nuclear, and cytoplasmic proteins were detected by immunoblotting using corresponding antibodies. The PEPC and H3 proteins were used as cytosolic and nuclear markers, respectively.

Results

P0BrA interacts with NbRAF2

To study the function of the P0 protein, we performed a Y2H screen of an Arabidopsis cDNA library using P0BrA as the bait to identify P0BrA-interacting proteins. Among the screened positive clones, one contained an intact ORF that shared 100% identity with AtRAF2 (At5g51110; GenBank accession AED96036.1). To explore the role of interactions between P0BrA and RAF2 in N. benthamiana, we searched for
AtRAF2 homologs in the *N. benthamiana* genome (http://solgenomics.net). The homolog that we identified encoded a 211-amino acid protein, shared approximately 50% identity with AtRAF2 and ZmRAF2 (GenBank accession NP_001144391) (see Supplementary Fig. S1), and was named NbRAF2 (GenBank accession MG560271). However, the traditional directed Y2H assay did not reveal a P0BrA-NbRAF2 interaction, although it did detect AtRAF2–P0BrA (Supplementary Fig. S2). Hence, we used the split ubiquitin MBY2H system to test for an interaction (McGee, 2006; Brückner et al., 2009). The P0BrA was cloned into either the MBY2H bait vector to express a TF::Cub::P0BrA (P0BrA–Cub)-fusion protein or into the MBY2H prey vector to express a NubG::P0BrA (NubG–P0BrA)-fusion protein. NbRAF2 was also cloned into either the MBY2H bait vector to express a TF::Cub::NbRAF2 (NbRAF2–Cub)-fusion protein or into the MBY2H prey vector to express a NubG::NbRAF2 (NubG–NbRAF2)-fusion protein. A series of controls were performed. NubG constructs served as test proteins and Nubl constructs were used as positive controls. All combinations were transformed into yeast cells for mating, and the transformed yeast cells were transferred to S/−WL and S/−AHLW plates for 2–3 d. However, the NubG–NbRAF2/Cub combination also grew on the S/−AHLW selective plates (data not shown). Thus, we chose NbRAF2–Cub to identify the interaction. Yeast transformed with NbRAF2–Cub and NubG–P0BrA grew on selective plates, as did the positive control; however, the negative controls did not (Fig. 1A). Thus, P0BrA can directly interact with NbRAF2 in yeast.

**Fig. 1.** P0BrA interacts with NbRAF2. (A) Analysis of interactions between NbRAF2 and P0BrA using the split ubiquitin membrane-bound yeast two-hybrid (MBY2H) system. NbRAF2 was cloned into the MBY2H bait vector pBT3-STE, and P0BrA was cloned into the prey vector pPR3-N. NubG constructs served as test proteins and Nubl constructs were positive controls. All the combinations were transformed into yeast NMY51. Yeast strains were grown on S/−Leu/−Trp and S/−Ade/−His/−Leu/−Trp, and maintained at 30 °C for 2–3 d. (B) Co-immunoprecipitation analyses of NbRAF2 and P0BrA proteins in *N. benthamiana* leaves. P0BrA-Flag was co-expressed with GFP, NbRAF2–GFP, or NbSKP1–GFP through agro-infiltration. GFP and NbSKP1 were used as negative and positive controls, respectively. Protein complexes were immunoprecipitated using anti-Flag beads. Immunoprecipitates were assessed with western blotting using anti-GFP and anti-Flag antibodies.

We used Co-IP assays to further confirm whether P0BrA interacted with NbRAF2 in plant cells. Flag-tagged P0BrA (P0BrA–Flag) was co-expressed with GFP, GFP-tagged NbRAF2 (NbRAF2–GFP), or GFP-tagged NbSKP1 (NbSKP1–GFP) in *N. benthamiana* leaves through agro-infiltration. The interaction between P0BrA and NbSKP1 was used as a positive control. At 2 dpi, protein complexes were immunoprecipitated using anti-Flag beads. The P0BrA–Flag was co-immunoprecipitated with NbRAF2–GFP, but not with GFP (Fig. 1B). These experiments demonstrated that P0BrA interacts with NbRAF2 in plant cells.

**Subcellular localization of NbRAF2**

To investigate the subcellular localization of NbRAF2, we generated mCherry-tagged NbRAF2 (NbRAF2–mCherry) (see Supplementary Fig. S3), and then transiently co-expressed it with free GFP in *N. benthamiana* leaves through agro-infiltration. The infiltrated leaves were collected at 2 dpi and observed with confocal microscopy. The mCherry signal revealed that NbRAF2 was present in the chloroplast, cell periphery, and nucleus of the same cells, similar to the subcellular localization of AtSDIRIP1/AtRAF2. Surprisingly, NbRAF2 was localized to the chloroplast stromules (Fig. 2A). To confirm this, we transiently expressed NbRAF2–mCherry in FNR–EGFP transgenic *N. benthamiana* through agro-infiltration. The FNR–EGFP was used here as a stromule-localized marker (Schattat et al., 2011). Confocal microscopy showed that NbRAF2–mCherry could perfectly co-localize with FNR–EGFP in chloroplasts and stromules (Fig. 2B). Thus, NbRAF2–mCherry was localized not only to the nucleus and cell periphery but also to the chloroplasts and stromal fraction. Similarly, AtRAF2 was also localized to the stromules (Supplementary Fig. S4).

**The C-terminal residues of NbRAF2 are required for its self-interaction**

The Y2H and BiFC assays showed that NbRAF2 self-interacted. The Y2H results demonstrated that the combinations of pGBK7–NbRAF2 and pGADT7–NbRAF2 could not grow on the S/−AHLW selective plates (Fig. 3A), but NbRAF2 could interact with NbRAF2–ΔTP, a truncated mutant of NbRAF2 that contained a deleted transit peptide (TP) of 1–45 aa in the N-terminus (Figs 2, 3A, Supplementary Fig. S3). For the BiFC assays, NbRAF2 was fused to N- and C-terminal halves of YFP at the N-terminus of AtSDIRIP1/AtRAF2. Surprisingly, NbRAF2 was co-localized with the chloroplast stromules (Fig. 2). To confirm this, we transiently expressed NbRAF2–mCherry and NbSKP1–GFP in N/−AHLW plates (data not shown). Thus, we chose NbRAF2–Cub to identify the interaction. Yeast transformed with NbRAF2–Cub and NubG–P0BrA grew on selective plates, as did the positive control; however, the negative controls did not (Fig. 1A). Thus, P0BrA can directly interact with NbRAF2 in yeast.

**The C-terminal residues of NbRAF2 are required for its self-interaction**

The Y2H and BiFC assays showed that NbRAF2 self-interacted. The Y2H results demonstrated that the combinations of pGBK7–NbRAF2 and pGADT7–NbRAF2 could not grow on the S/−AHLW selective plates (Fig. 3A), but NbRAF2 could interact with NbRAF2–ΔTP, a truncated mutant of NbRAF2 that contained a deleted transit peptide (TP) of 1–45 aa in the N-terminus (Figs 2, 3A, Supplementary Fig. S3). For the BiFC assays, NbRAF2 was fused to N- and C-terminal halves of YFP at the N-termini to generate NbRAF2–YN and NbRAF2–YC, respectively. The reconstituted YFP fluorescence was observed in *N. benthamiana* leaf epidermal cells co-infiltrated with NbRAF2–YN and NbRAF2–YC, which co-localized with chloroplast auto-fluorescence (Fig. 3B). In addition, YFP signals occurred in the stromules. Fluorescence could not be visualized in the negative controls NbRAF2–YN/YC and YN/NbRAF2–YC (Supplementary Fig. S5). Arabidopsis and maize RAF2 can form dimers (Valkai, 2004; Feiz et al., 2014), consistent with our observations for NbRAF2. In addition, the C-terminal truncated mutant NbRAF2–ΔCter, which contained a deleted C-terminal 183–211 aa and localized to the nucleus
and chloroplast (Supplementary Fig. S3, Fig. 2), failed to interact with full-length NbRAF2 (Fig. 3A, B).

Thus, NbRAF2 self-interacted in chloroplasts and stromules, and the C-terminal residues were required for self-interaction but not for chloroplastic and nuclear localization. Similarly, the C-terminal 192–220 aa of AtRAF2 was required for its self-interaction but not for its chloroplastic and nuclear localization (Supplementary Figs S4, S6).
Sun et al. NbRAF2 co-localizes with P0\(^{BrA}\) in the nucleus and cell periphery

To investigate the subcellular localization of P0\(^{BrA}\), we constructed a vector expressing the P0\(^{BrA}\) protein with a GFP-tag fused to its C-terminus (P0\(^{BrA}\)-GFP). P0\(^{BrA}\)-GFP was co-expressed with free mCherry (which was used as a marker to delineate the nucleus and cytoplasm) in N. benthamiana leaves through agro-infiltration. Confocal microscopy showed that P0\(^{BrA}\)-GFP co-localized with mCherry, suggesting that P0\(^{BrA}\)-GFP can localize to the nucleus and the cell periphery (Fig. 4A). Moreover, P0\(^{BrA}\)-GFP also formed punctate structures in the cytosol (Fig. 4A). To further investigate where P0\(^{BrA}\) and NbRAF2 co-localized in vivo, the P0\(^{BrA}\)-GFP was transiently co-expressed with NbRAF2–mCherry in N. benthamiana leaves through agro-infiltration. Confocal microscopy showed that they co-localized in the nucleus and cell periphery (Fig. 4B).

The nuclear pool of NbRAF2 decreases in the presence of P0\(^{BrA}\) or BrYV-A

Although P0\(^{BrA}\)-GFP co-localized with NbRAF2–mCherry in the nucleus, the cells demonstrating nuclear co-localization were difficult to find. Interestingly, the distribution of NbRAF2–mCherry to the nucleus was significantly reduced in the presence of P0\(^{BrA}\)-GFP compared with the GFP control (Fig. 5A). The relative numbers of nuclear-localized NbRAF2 were calculated based on the confocal images, and there was a reduction of approximately 30% in nuclear-localized NbRAF2 in the P0\(^{BrA}\)-GFP co-expressed cells compared with GFP co-expressed cells. Nucleocytoplasmic fractionation assays were performed to further identify the nuclear accumulation of the NbRAF2 protein. The NbRAF2–mCherry with P0\(^{BrA}\)-GFP or GFP were transiently co-expressed in N. benthamiana leaves, and protein samples were...
P0 protein impairs NbRAF2 antiviral activity and aids virus infection | 3133

prepared at 2 dpi. A western blot analysis revealed that the nuclear pool of NbRAF2 protein significantly decreased after leaves were inoculated with P0BrA–GFP (Fig. 5B). However, no significant difference was observed in the total amounts of NbRAF2 protein.

NbRAF2–ΔTP, the TP deletion mutant, localized to the nucleus but not to the chloroplast; however, NbRAF2–ΔCter, the C-terminal residue deletion mutant, localized to the nucleus and the chloroplast (Fig. 2). We tested the effect of P0BrA on the nuclear accumulation of these two mutants. Confocal microscopy showed that P0BrA inhibited the accumulation of a nuclear pool of NbRAF2–ΔCter–mCherry, but not that of NbRAF2–ΔTP–mCherry (see Supplementary Fig. S7). Thus, the P0BrA-mediated reduction of nuclear NbRAF2 required the dual localization of NbRAF2 in the chloroplast and nucleus.

The effect of BrYV-A on the nuclear accumulation of NbRAF2 was assayed using nucleocytoplasmic fractionation assays and confocal microscopy. NbRAF2–mCherry was co-expressed with the mock or BrYV-A through agro-infiltration in N. benthamiana leaves. The samples were prepared from inoculated leaves at 2 dpi. The nuclear pool of NbRAF2 decreased after BrYV-A infection (Fig. 5C, Supplementary Fig. S8).

Fig. 4. NbRAF2 co-localizes with P0BrA in the nucleus and cell periphery. (A) P0BrA localizes to the nucleus and the cytoplasm. GFP-tagged P0BrA was transiently co-expressed with mCherry in N. benthamiana leaves. (B) Co-localization of NbRAF2 and P0BrA. The co-localization of the proteins is shown in the merged image. Confocal images show that NbRAF2 co-localizes with P0BrA in the nucleus and cell periphery. Images were taken at 2 d post-inoculation. (This figure is available in colour at JXB online.)

NbRAF2 expression is down-regulated during BrYV-A infection

We investigated whether the NbRAF2 expression level was affected during BrYV-A infection. Total RNA was obtained from BrYV-A-inoculated N. benthamiana leaves at 2 dpi. Semi-quantitative RT-PCR showed that the mRNA level of NbRAF2 decreased during the BrYV-A infection (Fig. 6A).

Silencing NbRAF2 increases the local accumulation level of BrYV-A in inoculated leaves and enhances systemic infection of the virus

To investigate the possible role of NbRAF2 during BrYV-A infection, we used the TRV-VIGS vector (Liu et al., 2002b) to knock down the expression of NbRAF2. A partial cDNA fragment of NbRAF2 was cloned into the RNA2-derived vector of TRV to generate pTRV2–NbRAF2, and GFP was cloned into pTRV2 to generate pTRV2–GFP, which was used as a negative control. At 2-3 weeks post-inoculation, NbRAF2-silenced plants showed leaf chlorosis compared with TRV–GFP-infected control plants (Fig. 6B). The NbRAF2 expression level was then determined with RT-PCR. The NbRAF2 mRNA level was significantly reduced in NbRAF2-silenced plants compared with non-silenced controls (Fig. 6C). The RbcL protein level correspondingly decreased in NbRAF2-silenced plants (Fig. 6D) and RbcL interacted with NbRAF2 (see Supplementary Fig. S9), consistent with the role of ZmRAF2 and AtRAF2 in Rubisco accumulation (Feiz et al., 2014; Fristedt et al., 2018). Subsequently, the NbRAF2-silenced and non-silenced N. benthamiana plants were inoculated with BrYV-A using agro-infiltration. Western blot and RT-PCR analyses showed that BrYV-A CP protein and BrYV-A RNA levels increased in the NbRAF2-silenced plants compared with non-silenced control plants at 2 dpi (Fig. 6D). BrYV-A can cause necrosis in inoculated leaves, but symptoms are not visible in systemic leaves. However, we observed no obvious
difference in necrosis of *NbRAFT*-silenced and non-silenced plants in the inoculated leaves at 3 and 4 dpi (Supplementary Fig. S10). At 14 dpi, BrYV-A was able to spread to the upper non-inoculated leaves of the *NbRAFT*-silenced plants but not easily into those of non-silenced plants. BrYV-A RNA was detected in the systemic leaves of *NbRAFT*-silenced and non-silenced plants (Fig. 6E). RT-PCR detection showed that 31.6% of non-silenced control plants had systemic BrYV-A infection at 14 dpi, but 63.2% of *NbRAFT*-silenced plants became systemically infected with BrYV-A (Fig. 6E, lower panel). These findings suggested that silencing of *NbRAFT* increases the local accumulation of BrYV-A in inoculated leaves and promotes the systemic infection of the virus. Thus, our results showed that NbRAFT negatively regulates the BrYV-A infection.
Overexpression of nuclear RAF2 enhances resistance to BrYV-A

Silencing NbRAFT increased BrYV-A accumulation, and the nuclear accumulation of NbRAFT decreased during BrYV-A infection (Figs 5c, 6d). Therefore, we investigated the function of nuclear NbRAFT in BrYV. A nuclear localization sequence (NLS; Wen et al., 1995) was fused to the N-terminus of NbRAFT–ΔTP–mCherry to generate NLS–NbRAFT–ΔTP–mCherry (see Supplementary Fig. S3). Confocal microscopy showed that NLS–NbRAFT–ΔTP–mCherry
was exclusively redirected to the nucleus (Fig. 7A). Hence, we transiently expressed NLS–NbRAF2–ΔTP–mCherry and mCherry through agro-infiltration in *N. benthamiana* leaves. Then, at 1 dpi, we inoculated BrYV-A through agro-infiltration in the same leaves for 2 d. The accumulation of BrYV-A CP was examined with western blotting, which demonstrated that overexpressing nuclear NbRAF2 decreased the level of BrYV-A CP compared with mCherry (Fig. 7B). To determine whether nuclear AtRAF2 had the same role in resistance to BrYV-A, we constructed AtRAF2–ΔTP–mCherry, which was exclusively redirected to the nucleus (Supplementary Fig. S11). The results of western blotting demonstrated that overexpressing nuclear AtRAF2 also inhibited virus accumulation (Supplementary Fig. S11). These results suggested that the increased nuclear accumulation of NbRAF2 or AtRAF2 enhances resistance to BrYV-A.

**Discussion**

We screened a polerovirus P0-interacting chloroplast/nucleus protein, NbRAF2, and found that it was localized not only to the cell periphery and nucleus but also to the chloroplasts and stromules. In addition, BiFC and Y2H assays showed that NbRAF2 could self-interact in chloroplasts and stromules, and the self-interaction required the 183–211 aa of its C-terminal. We obtained similar results for AtRAF2.

The C-terminus of the RAF2 protein is highly conserved in *N. benthamiana*, *A. thaliana*, and *Z. mays*, indicating that the C-terminus and self-interaction may play important roles in the functions of plant RAF2 proteins. NbRAF2 interacted with NbRbcL, and *NbRAF2*-silenced plants showed reduced accumulation of NbRbcL, demonstrating that NbRAF2 may play a role similar to ZmRAF2 and AtRAF2 in Rubisco assembly (Feiz *et al.*, 2014; Fristedt *et al.*, 2018).

Increasing evidence indicates that several chloroplast proteins are localized in both chloroplasts and nuclei. These proteins have pivotal roles in plastid-to-nucleus communication (retrograde signaling), which can regulate specific changes in nuclear gene expression, including photosynthesis-related and stress-responsive genes (Koussevitzky *et al.*, 2007; Pogson *et al.*, 2008; Krause *et al.*, 2012). In response to retrograde signals, PTm, a chloroplast envelope-bound plant homeodomain PHD transcription factor, is cleaved. Its N-terminus is transported through the cytosol to the nucleus, and there it activates *ABI4* transcription in a PHD-dependent manner to regulate *Lhcb* expression (Sun *et al.*, 2011). Several proteins with dual localization in the plastids and the nucleus are involved in pathogen defense reactions (Desveaux *et al.*, 2000, 2005; Caplan *et al.*, 2008; Lai *et al.*, 2011). For instance, after NRIP1 is released from the chloroplast to the cytoplasm and the nucleus during *Tobacco mosaic virus* infection, it is then recognized by the N immune receptor to activate defenses, and this is accompanied by the induction of the hypersensitive response (Caplan *et al.*, 2008). The Why1 protein is released from the chloroplast and transported to the nucleus where it acts as a nuclear transcription factor to mediate the elicitor-induced expression of the *PR-10a* gene (Desveaux *et al.*, 2000; Isemer *et al.*, 2012).

In our study, NbRAF2 showed chloroplast/nucleus dual localization, but the dual localization mechanism and the function of NbRAF2 in the nucleus remain unknown. Interestingly, we observed that NbRAF2 was also localized to the stromules. The stromules, from which chloroplasts send out dynamic tubular extensions, aid in the transport of immune signals into the nucleus and other subcellular compartments during innate immunity (Caplan *et al.*, 2015; Gu and Dong, 2015). Stromules can be induced during immune responses and are in close contact with nuclei (Kwok and Hanson, 2004; Holzinger *et al.*, 2007; Caplan *et al.*, 2008, 2015). We hypothesized that chloroplast/nucleus NbRAF2 may be transported from the chloroplasts to the nucleus through the stromules and may have a role in transcriptional programming in the nucleus, similar to other chloroplast/nuclear proteins.

We observed that P0BrA interacted and co-localized with NbRAF2 both in the nucleus and the cell periphery. The molecular mass of NbRAF2–mCherry corresponded to the predicted size of the mature NbRAF2–mCherry that was processed by the cleavage of the N-terminal chloroplast transit peptide (TP), and the nuclear isoform was the same size as the mature NbRAF2 protein, indicating that the nuclear NbRAF2 was released from chloroplasts and could interact with P0BrA. In addition, the subcellular distribution of NbRAF2 was altered by P0BrA. Confocal microscopy and the nucleocytoplasmic

---

**Fig. 7.** Overexpression of nuclear RAF2 enhances resistance to BrYV-A. (A) Confocal microscopy images showing the subcellular localization of mCherry (mC) and NLS–NbRAF2–ΔTP–mCherry (NLS–NbRAF2–ΔTP–mC). Images were taken at 2 d post-inoculation. The scale bars represent 10 μm. N, nucleus. (B) BrYV-A was agro-infiltrated at 1 dpi in leaves separately overexpressing NLS–NbRAF2–ΔTP–mC and mC. At 3 dpi, protein was extracted and subjected to western blotting. Coat protein (CP) was detected with BrYV-A CP polyclonal antiseraum. RbcL is the Rubisco large subunit. ImageJ software was used to quantify the bands. (This figure is available in colour at JXB online.)
fractionation assay showed that P0\textsuperscript{BrA}–GFP inhibited the accumulation of nuclear NbRAFT2. AtRAFT2 is degraded by the E3 ligases AtSDIR1 and AIRP2 in the cytosol when responding to ABA and high-salt stress (Zhang et al., 2015a; Oh et al., 2017). However, the total NbRAFT2 level was not affected when leaves were inoculated with P0\textsuperscript{BrA}, indicating that the reduction in the nuclear enrichment of NbRAFT2 was not caused by the degradation triggered by the F-box protein P0\textsuperscript{BrA}. Interestingly, P0\textsuperscript{BrA} decreased the nuclear pool of NbRAFT2–ΔCter, which localized to chloroplasts and nuclei, rather than the NbRAFT2–ΔTP, which localized to nuclei but not chloroplasts. This indicated that the P0\textsuperscript{BrA}-mediated reduction of nuclear NbRAFT2 requires the dual localization of NbRAFT2 in chloroplasts and nuclei. We hypothesize that the chloroplast-to-nucleus translocation of NbRAFT2 was regulated by chloroplast retrograde signals. Thus, P0\textsuperscript{BrA} interfered with the chloroplast retrograde signals to inhibit the chloroplast-to-nucleus translocation of NbRAFT2. As a result, P0\textsuperscript{BrA} inhibited the accumulation of nuclear NbRAFT2. It is unlikely that P0\textsuperscript{BrA} induced NbRAFT2 export from the nucleus to the cytoplasm and/or chloroplast through interactions with NbRAFT2 in the nucleus and cell periphery, because we observed no change in the nuclear pool of NbRAFT2–ΔTP when leaves were inoculated with P0\textsuperscript{BrA}. However, the mechanism remains elusive and will be further investigated in the future.

The nucleus of a eukaryotic cell is essential for controlling immune responses. Immune signals are transmitted to the nucleus and reprogram gene expression, which shifts the cells into defense mode (Maleck et al., 2000; Tao, 2003; Dodds and Rathjen, 2010). Upon pathogen infection, the nuclear pool of immune receptors increases or immune receptors are targeted to the nucleus where they activate defense responses (Deslandes et al., 2002, 2003; Shen et al., 2007; Bernoux et al., 2008). Other essential signaling components in the nucleus also play important roles in defense responses. The nuclear pool of the nucleo-cytoplasmic immune regulator EDS1 increases after a pathogen challenge and is essential for resistance to biotrophic and hemi-biotrophic pathogens (Garcia et al., 2010). Under unchallenged conditions, NPR1 is present in the cytosol as a stable oligomer (Mou et al., 2003); however, upon pathogen infection, monomeric forms of NPR1 are transferred to the nucleus to bind the TGA family of transcription factors, activating the expression of defense-related genes, such as PR1 and WRKY (Zhang et al., 1999; Mou et al., 2003; Wang et al., 2006). We found that the nuclear pool of NbRAFT2 decreased during BrYV-A infection and could negatively regulate BrYV-A infection. Silencing NbRAFT2 increased BrYV-A accumulation in the inoculated leaves and enhanced viral systemic infection. However, we cannot rule out the possibility that NbRAFT2 could also be involved in BrYV-A movement. In contrast, overexpression of nuclear NbRAFT2 inhibited BrYV-A accumulation in the inoculated leaves, indicating that nuclear NbRAFT2 possesses antiviral activity against BrYV-A infection. Interestingly, overexpressing nuclear NbRAFT2 enhanced the necrosis when inoculated with BrYV-A (Supplementary Fig. S12), which is consistent with our hypothesis that the increased resistance to BrYV-A is probably caused by the enhanced defense signaling when overexpressing RAF2 in the nucleus. Future work will address these issues. In mammals, PCD/DCoH, a homolog of plant RAF2, acts as a transcriptional co-factor to bind to, and enhance the activity of, the HNF1 transcription factor in the nucleus (Suck and Ficner, 1996). There is a good possibility that NbRAFT2 acts as a transcriptional co-factor in the nucleus to regulate defense response-related genes. Thus, P0\textsuperscript{BrA} may affect antiviral activity of NbRAFT2 through two pathways. One is that the nuclear P0 protein directly interacts with nuclear NbRAFT2 to interfere with its antiviral function; another is that P0\textsuperscript{BrA} inhibits the accumulation of the nuclear pool of NbRAFT2 by sequestering it outside the nucleus in order to enhance the viral infection. Moreover, P0 proteins encoded by PLRV, Melon aphid-borne yellows virus, and Sugarcane yellow leaf virus interacted with AtRAFT2 or NbRAFT2 (Supplementary Fig. S13), P0\textsuperscript{BrA} decreased the nuclear pool of NbRAFT2 (Supplementary Fig. S14), and overexpression of nuclear AtRAFT2 also inhibited BrYV-A accumulation. Thus, the host RAF2 protein might be a common target of polerovirus P0 proteins.

In light of our data, we hypothesize that, under normal conditions, NbRAFT2 forms dimers and participates in Rubisco assembly in the chloroplast. Nuclear NbRAFT2 functions as a transcriptional co-factor to regulate defense response-related genes. Upon pathogen infection, the inactive nuclear NbRAFT2 is activated to initiate expression of defensive genes. Meanwhile, chloroplasts sense the changes and transport chloroplastic immune signals, including NbRAFT2, to the nucleus through stomules or other pathways to enhance resistance to pathogens. However, the effector P0 protein interacts with NbRAFT2, thereby interfering with the antiviral function of nuclear NbRAFT2. Subsequently, P0\textsuperscript{BrA} inhibits NbRAFT2 nuclear accumulation by interfering with the chloroplast retrograde signals to amplify the suppression process for the the benefit of the virus, which further facilitates pathogen infection.

Supplementary data

Supplementary data are available at JXB online.

Table S1. List of primers used in this research.

Figure S1. Multiple sequence alignment of representative RAF2 proteins.

Figure S2. Yeast two-hybrid and co-immunoprecipitation analyses demonstrating that AtRAFT2 interacts with P0\textsuperscript{BrA}.

Figure S3. NbRAFT2, AtRAFT2, and their mutant constructs used for confocal microscopy or Y2H assays.

Figure S4. The subcellular localization of AtRAFT2 and AtRAFT2–ΔCter.

Figure S5. Controls for BiFC assays for NbRAFT2 self-interaction.

Figure S6. Self-interaction of AtRAFT2 as demonstrated by Y2H and BiFC assays.

Figure S7. P0\textsuperscript{BrA}–GFP decreases the nuclear pool of NbRAFT2–ΔCter but not NbRAFT2–ΔTP.

Figure S8. The localization of NbRAFT2–mCherry when co-expressed with BrYV-A.
Fig. S9. Yeast two-hybrid and co-immunoprecipitation analyses demonstrating that NbRAF2 interacts with NbRbcL.

Fig. S10. Images of leaves of wild-type, NbRAF2-silenced, and non-silenced *N. benthamiana* inoculated with BrYV-A.

Fig. S11. Overexpression of nuclear AtRAF2 enhances resistance to BrYV-A, as demonstrated by AtRAFT2–ΔTP–mCherry and western blotting.

Fig. S12. Images of leaves demonstrating that overexpression of nuclear NbRAF2 enhances necrosis when inoculated with BrYV-A.

Fig. S13. Yeast two-hybrid and co-immunoprecipitation analyses demonstrating that polerovirus P0 proteins interact with AtRAFT2 and NbRAFT2.

Fig. S14. P0PL decreases the nuclear enrichment of NbRAFT2, as demonstrated by western blotting.

### Acknowledgments

This work was supported by the National Natural Science Foundation of China (31671995, 31371909 and 31071663) and the 111 project (B13006). We thank Drs David Baulcombe (Department of Plant Sciences, University of Cambridge, UK) for providing the wild-type *N. benthamiana*, Andrew O. Jackson (Department of Plant and Microbial Biology, University of California, Berkeley, USA) for providing the vectors pGDD and pGDDG, Xiao-Rong Tao (Department of Plant Pathology, Nanjing Agricultural University, Nanjing, China) for providing the vector pCB301-2 × 3S-MCS-HDV*α*-NOS, Yu-Le Liu (School of Life Sciences, Tsinghua University, Beijing, China) for providing the VIGS vectors pTRV1 and pTRV2, Jaideath Mathur (Department of Molecular and Cellular Biology, University of Guelph, Canada) for providing the FNRI-GFP transgenic *N. benthamiana*, Shu-Hua Yang (China Agricultural University, Beijing) for providing the vector pSuper1300–GFP and Arabidopsis cDNA library, and Jun-Ping Gao (China Agricultural University, Beijing) for providing the split ubiquitin MbY2H system.

### References

Balasubramaniam M, Kim BS, Hutchens-Williams HM, Loesch-Fries LS. 2014. The photosystem II oxygen-evolving complex protein PsbP interacts with the coat protein of Alfalfa mosaic virus and inhibits virus replication. Plant Molecular-Plant Interactions 27, 1107–1118.

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.

Bernoix M, Timmers T, Jaunier A, Brière C, de Wit PJ, Marco Y, Deslandes L. 2008. RD19, an Arabidopsis thaliana plant–virus interaction. Molecular Plant Pathology 19, 1811–1818.

Bhattacharyya D, Chakraborty S. 2018. Chloroplast: the Trojan horse in plant–virus interaction. Molecular Plant Pathology 19, 504–518.

Bortolamiol D, Pazhouhandeh M, Marrocco K, Genschik P, Ziegler-Graff V. 2007. The Polerovirus P box protein P0 targets ARGONAUTE1 to suppress RNA silencing. Current Biology 17, 1615–1621.

Brückner A, Polge C, Lentze N, Auerbach D, Schlattner U. 2009. Yeast two-hybrid, a powerful tool for systems biology. International Journal of Molecular Sciences 10, 2763–2788.

Caplan JL, Kumar AS, Park E, Padmanabhan MS, Hoban K, Modla S, Czymmek K, Dinesh-Kumar SP. 2015. Chloroplast stromules function during innate immunity. Developmental Cell 34, 45–57.

Caplan JL, Mammillapalli P, Burch-Smith TM, Czymmek K, Dinesh-Kumar SP. 2008. Chloroplastic protein NRIIP1 mediates innate immune receptor recognition of a viral effector. Cell 132, 449–462.

Chen S, Jiang GZ, Wu JX, Liu Y, Qian YJ, Zhou XP. 2016. Characterization of a novel Polerovirus infecting maize in China. Viruses 8, 120.

Csorba T, Lózsa R, Huvátváger G, Burgýán J. 2010. Polerovirus protein P0 prevents the assembly of small RNA-containing RISC complexes and leads to degradation of ARGONAUTE1. The Plant Journal 62, 462–473.

Delfosse VG, Agrofoglio YC, Casse MF, Kresic IB, Hoop HE, Ziegler-Graff V, Distelfano AJ. 2014. The P0 protein encoded by cotton leafroll dwarf virus (CLRDV) inhibits local but not systemic RNA silencing. Virus Research 180, 70–75.

Derrien B, Baumberger N, Schepetilnikov M, Viotti C, De Cilia J, Ziegler-Graff V, Isomo E, Schumacher K, Genschik P. 2012. Degradation of the antiviral component ARGONAUTE1 by the autophagy pathway. Proceedings of the National Academy of Sciences, USA 109, 15942–15946.

Deslandes L, Olivier J, Peeters N, Feng DX, Khounlootham M, Boucher C, Sommisch I, Genin S, Marco Y. 2003. Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. Proceedings of the National Academy of Sciences, USA 100, 8024–8029.

Deslandes L, Olivier J, Thieulières F, Hirsch J, Feng DX, Bittner-Eddy P, Beynon J, Marco Y. 2002. Resistance to *Rhabdion solanacearum* in Arabidopsis thaliana is conferred by the recessive *RRS1-R* gene, a member of a novel family of resistance genes. Proceedings of the National Academy of Sciences, USA 99, 2404–2409.

Desveaux D, Després C, Joyeux A, Subramanian R, Brisson N. 2000. PBF-2 is a novel single-stranded DNA binding factor implicated in *PR-10a* gene activation in tobacco. The Plant Cell 12, 1477–1489.

Desveaux D, Maréchal A, Brisson N. 2005. Whirly transcription factors: defense gene regulation and beyond. Trends in Plant Science 10, 95–102.

Dodd PN, Rathjen JP. 2010. Plant immunity: towards an integrated view of plant-pathogen interactions. Nature Reviews Genetics 11, 539–548.

Feiz L, Williams-Carrier R, Belcher S, Montano M, Barkan A, Stern DB. 2014. A protein with an inactive pterin-4a-carbinolamine dehydratase domain is required for Rubisco biogenesis in plants. The Plant Journal 80, 862–869.

Fristedt R, Hu C, Wheatley N, et al. 2018. RAF2 is a Rubisco assembly factor in Arabidopsis thaliana. The Plant Journal 94, 146–156.

Fusaro AF, Correa RL, Nakasugi K, Jackson C, Kawchuk L, Vaslin MF, 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.

García AV, Blanvillain-Baufumé S, Hulbers RP, Wiermer M, Li G, Gobbato E, Rietz S, Parker JE. 2010. Balanced nuclear and cytoplasmic activities of EDS1 are required for a complete plant innate immune response. PLoS Pathogens 6, e1000970.

Goodin MM, Dietzgen RG, Schichnes D, Ruzin S, Jackson AO. 2002. pGD vectors: versatile tools for the expression of green and red fluorescent protein fusions in agroinfiltrated plant leaves. The Plant Journal 31, 375–383.

Gu Y, Dong X. 2015. Stromules: signal conduits for plant immunity. Developmental Cell 34, 3–4.

Han YH, Xiang HY, Wang Q, Li YY, Wu WQ, Han GC, Li DW, Yu JL. 2010. Ring structure amino acids affect the suppressor activity of *Malon aphid-borne yellows virus* P0 protein. Virology 406, 21–27.

Holsters M, de Waele D, Depicker A, Messens E, van Montagu SC, Robberecht W. 1997. Transfection and transformation of *Agrobacterium tumefaciens*. Molecular & General Genetics 163, 181–187.

Holzinger A, Buchner O, Lütz C, Hanson MR. 2007. Temperature-sensitive formation of chloroplast protrusions and stromules in mesophyll cells of Arabidopsis thaliana. Protoplasma 230, 23–30.

Isemr R, Mulisch M, Schäfer A, Kirchner S, Koop HU, Krupinska K. 2012. Recombinant Whirly1 translocates from transplastomic chloroplasts to the nucleus. FEBS Letters 586, 85–88.

Jiménez I, López L, Alamillo JM, Valli A, García JA. 2006. Identification of a *Pum pox virus* CI-interacting protein from chloroplast that has a negative effect on virus infection. Molecular Plant-Microbe Interactions 19, 350–358.

Jin Y, Ma D, Dong J, Li D, Deng C, Jin J, Wang T. 2007. The HC-pro protein of *Potato virus Y* interacts with NtMINd of tobacco. Molecular Plant-Microbe Interactions 20, 1505–1511.

Kong L, Wu J, Lu L, Xu Y, Zhou X. 2014. Interaction between *Rice stripe virus* disease-specific protein and host PsbP enhances virus symptoms. Molecular Plant 7, 691–708.
Koussevitzky S, Nott A, Mockler TC, Hong F, Sachetto-Martins G, Surpin M, Lim J, Mittler R, Chory J. 2007. Signals from chloroplasts converge to regulate nuclear gene expression. Science 316, 715–719.

Kozlowska-Makulaska A, Guillely H, Szynel MS, Beuve M, Lemaire O, Herrbach E, Bouzoubaa S. 2010. Pol proteins of European beet-infecting poleroviruses display variable RNA silencing suppression activity. The Journal of General Virology 91, 1082–1091.

Krause K, Oetke S, Krupinska K. 2012. Dual targeting and retrograde translocation: regulators of plant nuclear gene expression can be sequestered by plastids. International Journal of Molecular Sciences 13, 11085–11101.

Kwok EY, Hanson MR. 2004. Plastids and stromules interact with the nucleus and cell membrane in vascular plants. Plant Cell Reports 23, 188–195.

Lai Z, Li Y, Wang F, Cheng Y, Fan B, Yu JQ, Chen Z. 2011. Arabidopsis sigma factor binding proteins are activators of the WRKY33 transcription factor in plant defense. The Plant Cell 23, 3824–3841.

Liu Y, Schiff M, Dimash-Kumar SP. 2002a. Virus-induced gene silencing in tobacco. The Plant Journal 31, 777–786.

Liu Y, Schiff M, Marathe R, Dinesh-Kumar SP. 2002b. Tobacco Rar1, EDS1 and NPR1/NIMI1 like genes are required for N-mediated resistance to Tobacco mosaic virus. The Plant Journal 30, 415–429.

Malek K, Levine A, Eulgem T, Morgan A, Schmid J, Lawton KA, Dangl JL, Dietrich RA. 2000. The transcriptome of Arabidopsis thaliana during systemic acquired resistance. Nature Genetics 26, 403–410.

Mangwende T, Wang ML, Borth W, Hu J, Moore PH, Mirkov TE, Albert HH. 2009. The Pol gene of Sugarcane yellow leaf virus encodes an RNA silencing suppressor with unique activities. Virology 384, 38–50.

McGee MD, Rillo R, Anderson AS, Starr DA. 2006. UNC-83 is a KASH protein required for nuclear migration and is recruited to the outer nuclear membrane by a physical interaction with the SUN protein UNC-84. Molecular Biology of the Cell 17, 1790–1801.

Mou Z, Fan W, Dong X. 2003. Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell 113, 935–944.

Napolinelli V, Noirel A, Ziemen M, et al. 2008. Phylogenomic and functional analysis of pterin-4a-carbinolamine dehydratase family (CCG2154) proteins in plants and microorganisms. Plant Physiology 146, 1515–1527.

Oh TR, Kim JH, Cho SK, Ryu MY, Yang SW, Kim WT. 2017. AATIRP2 E3 ligase affects ABA and high-salinity responses by stimulating its ATP1/SDIRIP1 substrate turnover. Plant Physiology 174, 2515–2531.

Pazhouhandeh M, Dieterle M, Marrocco K, et al. 2006. F-box-like domain in the polerovirus protein P0 is required for silencing suppressor function. Proceedings of the National Academy of Sciences, USA 103, 1994–1999.

Peter KA, Gildow F, Palukaitis P, Gray SM. 2009. The C terminus of the polerovirus P5a readthrough domain limits virus infection to the phloem. Journal of Virology 83, 5419–5428.

Pfeffer S, Dunoyer P, Heim F, Richards KE, Jonard G, Ziegler-Graff V. 2002. P0 of Beet western yellows virus is a suppressor of posttranscriptional gene silencing. Journal of Virology 76, 6815–6824.

Pogson BJ, Woo NS, Forster B, Small ID. 2008. Plastid signalling to the nucleus and beyond. Trends in Plant Science 13, 602–609.

Rhee KH, Stier G, Becker PB, Suck D, Ficner R. 1996. Structure and function of PCD/DCoH, an RNA silencing-suppressing factor. Virology 216, 351–361.

Saccomani SI, Lockhart BE. 2000. Transmission, characterization, and functional analysis of pterin-4a-carbinolamine dehydratase family (CCG2154) proteins in plants and microorganisms. Plant Physiology 146, 1515–1527.

Shi Y, Chen J, Hong X, Chen J, Adams MJ. 2007. A potyvirus P1 protein interacts with the Risksa Fe/S protein of its host. Molecular Plant Pathology 8, 785–790.

Stevens M, Freeman B, Liu HY, Herrbach E, Lemaire O. 2005. Beet poleroviruses: close friends or distant relatives? Molecular Plant Pathology 6, 1–9.

Suck D, Ficner R. 1996. Structure and function of PCD/DCoH, an enzyme with regulatory properties. FEBS Letters 389, 35–39.

Sun X, Feng P, Xu X, Guo H, Ma J, Chi W, Lin R, Lu C, Zhang L. 2011. A chloroplast envelope-bound PHD transcription factor mediates chloroplast signals to the nucleus. Nature Communications 2, 477.

Taliansky M, Mayo MA, Barker H. 2003. Potato leafroll virus: a classic pathogen shows some new tricks. Molecular Plant Pathology 4, 81–89.

Tao Y, Xie Z, Chen W, Glazebrook J, Chang HS, Han B, Zhu T, Zou G, Katagiri F. 2003. Quantitative nature of Arabidopsis responses during compatible and incompatible interactions with the bacterial pathogen Pseudomonas syringae. The Plant Cell 15, 317–330.

Valkai I. 2004. 3D, a new component of the phytochrome B signal transduction, in Arabidopsis thaliana. Acta Biologica Szegediensis 48, 87.

Wang D, Amornsiripanich N, Dong X. 2006. A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathogens 2, e123.

Wang KD, Emplefer R, Nguyen TT, Moffett R, Sozzi MA. 2015. Elicitation of hypersensitive responses in Nicotiana glutinosa by the suppressor of RNA silencing protein P0 from poleroviruses. Molecular Plant Pathology 16, 435–448.

Wen W, Meinloth JL, Tsien RY, Taylor SS. 1995. Identification of a signal for rapid export of proteins from the nucleus. Cell 82, 463–473.

Win J, Kamoun S, Jones AM. 2011. Purification of effector-target protein complexes via transient expression in Nicotiana benthamiana. Methods in Molecular Biology 712, 181–194.

Xiang HY, Dong SW, Shang QX, Zhou CJ, Li DW, Yu JL, Han CG. 2011. Molecular characterization of two genotypes of a new polerovirus infecting brassicas in China. Archives of Virology 156, 2251–2255.

Xiang HY, Han CG. 2011. Molecular characterization of novel poleroviruses and poleroviral P0 protein functional analysis. Acta Phytopathologica Sinica 41 (4), 177–178 (in Chinese).

Yang H, Shi Y, Liu J, Guo L, Zhang X, Yang S. 2010. A mutant CHS3 protein with TIR-NB-LRR-LIM domains modulates growth, cell death and freezing tolerance in a temperature-dependent manner in Arabidopsis. The Plant Journal 63, 283–296.

Yao M, Zhang T, Tian Z, Wang Y, Tao X. 2011. Construction of Agrobacterium-mediated Cucumber mosaic virus infectious cDNA clones and 2b deletion viral vector. Scientia Agricultura Sinica 44, 4886–4890.

Zhang H, Cui F, Wu Y, Lou L, Liu L, Tian M, Ning Y, Shu K, Tang S, Xie Q. 2015a. The RING finger ubiquitin E3 ligase SDIR1 targets SDIR1-INTERACTING PROTEIN1 for degradation to modulate the salt stress response and ABA signaling in Arabidopsis. The Plant Cell 27, 214–227.

Zhang XY, Dong SW, Xiang HY, Chen XR, Li DW, Yu JL, Han CG. 2011. Molecular characterization of two genotypes of a new polerovirus infecting brassicas in China. Archives of Virology 156, 2251–2255.

Zhang H, Cui F, Wu Y, Lou L, Liu L, Tian M, Ning Y, Shu K, Tang S, Xie Q. 2015b. The RING finger ubiquitin E3 ligase SDIR1 targets SDIR1-INTERACTING PROTEIN1 for degradation to modulate the salt stress response and ABA signaling in Arabidopsis. The Plant Cell 27, 214–227.

Zhao J, Zhang Z, Xiao Y, Liu Y, Liu Y. 2016. Chloroplast in plant–virus interaction. Frontiers in Microbiology 7, 1565.

Zhu T, Li Y, Xiang HY, Wu ZY, Wang XB, Wang Y, Zhang YL, Li DW, Yu JL, Han CG. 2014. Amino acid sequence motifs essential for P0-mediated suppression of RNA silencing in an isolate of Potato leafroll virus from Inner Mongolia. Molecular Plant-Microbe Interactions 27, 515–527.

Zybaikov B, Rutorsch H, Friso G, Rudella A, Emanuelsen O, Sun Q, van Wijk KJ. 2008. Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. PLoS ONE 3, e1994.