

# *Pseudomonas* HopU1 modulates plant immune receptor levels by blocking the interaction of their mRNAs with GRP7

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**Pathogens target important components of host immunity to cause disease. The *Pseudomonas syringae* type III-secreted effector HopU1 is a mono-ADP-ribosyltransferase required for full virulence on *Arabidopsis thaliana*. HopU1 targets several RNA-binding proteins including GRP7, whose role in immunity is still unclear. Here, we show that GRP7 associates with translational components, as well as with the pattern recognition receptors FLS2 and EFR. Moreover, GRP7 binds specifically FLS2 and EFR transcripts *in vivo* through its RNA recognition motif. HopU1 does not affect the protein–protein associations between GRP7, FLS2 and translational components. Instead, HopU1 blocks the interaction between GRP7 and FLS2 and EFR transcripts *in vivo*. This inhibition correlates with reduced FLS2 protein levels upon *Pseudomonas* infection in a HopU1-dependent manner. Our results reveal a novel virulence strategy used by a microbial effector to interfere with host immunity.**

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## Introduction

An important aspect of innate immunity is the perception of pathogen-associated molecular patterns (PAMPs) by specific

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pattern recognition receptors (PRRs) leading to PAMP-triggered immunity (PTI; Dodds and Rathjen, 2010; Segonzac and Zipfel, 2011). In *Arabidopsis thaliana*, the leucine-rich repeat receptor kinases (LRR-RKs) FLS2 and EFR recognize bacterial flagellin (or its derived peptide flg22) and EF-Tu (or its derived peptide elf18), respectively (Gomez-Gomez and Boller, 2000; Zipfel *et al.*, 2006), while perception of fungal chitin depends on the LysM-RK CERK1 (Miya *et al.*, 2007; Wan *et al.*, 2008). In addition to its role in chitin perception, CERK1 is required for peptidoglycan perception (Willmann *et al.*, 2011). Perception of flg22, elf18 or chitin induces a series of early immune responses, including a rapid burst of reactive oxygen species (ROS), phosphorylation events, gene expression, as well as late responses such as callose deposition at the plant cell wall and resistance to pathogens (Segonzac and Zipfel, 2011). Plant PRRs are key to immunity, as their inhibition or loss of function leads to enhanced susceptibility to adapted and non-adapted pathogens (Segonzac and Zipfel, 2011; Willmann *et al.*, 2011).

Pathogens must block or avoid PTI to cause disease. A potent strategy to inhibit PTI is via the action of secreted effectors delivered into the host cells leading to effector-triggered susceptibility (ETS; Dodds and Rathjen, 2010). The genome of the phytopathogenic bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000) encodes >30 type III-secreted effectors (T3SEs). Recently, several T3SEs from different *P. syringae* strains were shown to be virulence factors. Corresponding host targets have been identified only for a few of them, but they revealed that T3SEs interfere with key components of PTI (Block and Alfano, 2011). For instance, AvrPto is a kinase inhibitor that blocks PTI signalling by interfering with the activation of PRRs and/or the complex formation of PRRs with their associated proteins (Xing *et al.*, 2007; Shan *et al.*, 2008; Xiang *et al.*, 2008, 2011). AvrPtoB is a multifunctional protein carrying E3 ubiquitin ligase activity that leads to the degradation of several PRRs and the inhibition of the PRR-associated LRR-RK BAK1 (Gohre *et al.*, 2008; Shan *et al.*, 2008; Gimenez-Ibanez *et al.*, 2009; Cheng *et al.*, 2011; Zeng *et al.*, 2012). Other *Pto* DC3000 T3SEs target signalling components downstream of PRR activation (Block and Alfano, 2011; Feng *et al.*, 2012).

The *Pto* DC3000 T3SE HopU1 is a mono-ADP-ribosyltransferase (mono-ADP-RT) required for full virulence in *Arabidopsis* (Fu *et al.*, 2007). Ectopic expression of HopU1 in *A. thaliana* suppresses callose deposition induced by flg22 in a manner dependent on its mono-ADP-RT activity (Fu *et al.*, 2007). HopU1 targets at least five different *A. thaliana* RNA-binding proteins (RBPs), including Glycine-Rich Protein 7 (GRP7) and GRP8 (Fu *et al.*, 2007). HopU1 mono-ADP-ribosylates an arginine at position 49 (R49) located in the conserved ribonucleoprotein consensus sequence 1 (RNP-1) motif of the RNA recognition motif (RRM) of GRP7, and this modification affects GRP7's ability to bind RNA *in vitro*

(Jeong *et al*, 2011). Although HopU1 targets several RBPs, *grp7* null mutant plants produce less ROS and callose in response to flg22, elf18 and chitin (Fu *et al*, 2007; Jeong *et al*, 2011), indicating that GRP7 regulates both early and late PAMP responses. In addition, *grp7* plants are more susceptible to *Pto* DC3000 (Fu *et al*, 2007; Jeong *et al*, 2011). A mutation in R49 blocks the ability of GRP7 to complement these phenotypes (Jeong *et al*, 2011). These results demonstrate the importance of GRP7 in plant innate immunity and the potency of mono-ADP-ribosylation to block GRP7 function. However, as in the case for many targets of pathogenic effectors, the exact role of GRP7 in innate immunity and therefore the molecular mechanism underlying PTI suppression by HopU1 are still unclear.

Here, we illustrate a function for GRP7 in PTI that is inhibited by HopU1. We show that GRP7 associates with translational components *in vivo*, and more surprisingly that GRP7 associates with the PRRs FLS2 and EFR. However, HopU1 does not affect the associations between GRP7, FLS2 and translational components. Instead, we reveal that GRP7 binds *FLS2* and *EFR* mRNA *in vivo*, and that HopU1 blocks this interaction. The *FLS2* and *EFR* transcripts provide the first examples of *in vivo* targets for GRP7 with a clear biological function. This inhibition correlates with reduced FLS2 protein levels *in planta* upon infection with *Pto* DC3000 in a HopU1-dependent manner. Our results reveal a novel virulence strategy used by a microbial effector to interfere with host immunity.

## Results

### **Modulation of GRP7 level and activity affects early and late immune responses**

Previous results conclusively showed that loss of GRP7 impairs PTI and resistance to *Pto* DC3000 infection (Fu *et al*, 2007; Jeong *et al*, 2011). To investigate the consequences of ectopic GRP7 expression, we monitored PTI and pathogen response in transgenic *A. thaliana* plants expressing untagged GRP7 under the control of the constitutive promoter 35S (GRP7ox lines) (Streitner *et al*, 2008). An immunoblot analysis using a specific anti-GRP7 antibody confirmed higher GRP7 levels in transgenic homozygous GRP7ox plants in comparison to the wild-type (WT) Col-2 ecotype (Supplementary Figure S1). *A. thaliana* Col-2 (WT) and GRP7ox plants were treated with flg22, elf18 or chitin, which resulted in substantially higher ROS production in GRP7ox plants compared to WT (Figure 1A). Similarly, callose deposition was increased in GRP7ox plants compared to WT plants after all three treatments (Figure 1B).

The *A. thaliana* GRP7ox plants were used in pathogenicity assays with *Pto* DC3000 or the *Pto* DC3000 *hrcC*<sup>-</sup> mutant that does not secrete any T3SEs and is therefore severely hypo-virulent. Plants were spray inoculated and bacteria were enumerated at 0 and 4 days after inoculation. Interestingly, GRP7ox plants were more resistant to infection by *Pto* DC3000 than WT plants (Figures 1C and D). The *Pto* DC3000 *hrcC*<sup>-</sup> mutant exhibited unaltered growth on GRP7ox plants. This may be due to the strongly reduced virulence of the *Pto* DC3000 *hrcC*<sup>-</sup> mutant to which the endogenous GRP7 seems to be sufficient to confer high resistance. The increased resistance to *Pto* DC3000 infection observed in plants

overexpressing GRP7 clearly demonstrates its important role in innate immunity.

GRP7 is required for full immunity to *Pto* DC3000 WT and *hrcC*<sup>-</sup>, and HopU1 targets GRP7 (Fu *et al*, 2007; Jeong *et al*, 2011). To assess the extent to which HopU1 inhibits PTI responses, we analysed early and late responses triggered by flg22 in transgenic *A. thaliana* lines constitutively expressing HopU1 C-terminally tagged with haemagglutinin (HA) under the control of the 35S promoter (Fu *et al*, 2007). In HopU1 plants, the ROS burst induced by flg22 and elf18 treatment was reduced compared to WT plants (Supplementary Figure S2A). Next, we confirmed that HopU1 leaves exhibit less callose deposition upon flg22 treatment (Supplementary Figure S2B; Fu *et al*, 2007). The reduced flg22 responsiveness of the HopU1 plants correlated with reduced FLS2 protein levels observed in three out of four independent biological experiments (Supplementary Figure S2C). These results were further validated using *A. thaliana* transgenic lines expressing HopU1-HA under the control of an estradiol-inducible promoter (*ind\_HopU1*) (Supplementary Figures S2D and E). Together, this demonstrates that HopU1 affects both early and late flg22-induced responses.

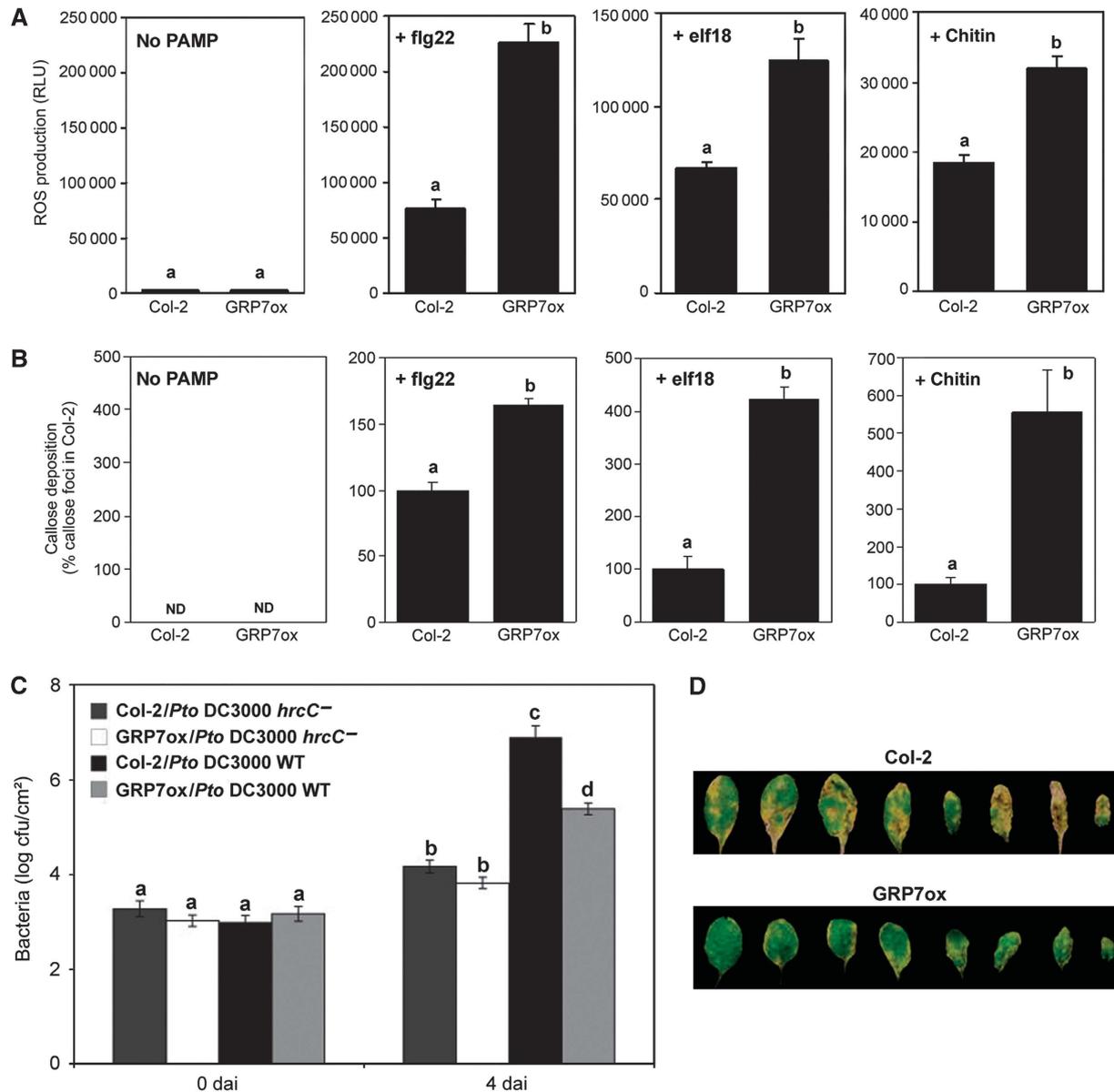
To test whether *in planta* HopU1 expression affects *A. thaliana* disease resistance, we assayed bacterial growth after spray inoculation with the *Pto* DC3000 strains WT, *hrcC*<sup>-</sup> or  $\Delta$ *hopU1* that is hypo-virulent (Fu *et al*, 2007). HopU1 plants were more susceptible to all the strains tested (Supplementary Figure S2F), albeit to a lesser extent than *fls2* null mutant plants consistent with the reduced flg22 sensitivity of HopU1 plants (Supplementary Figures S2A–E). Consistent with the notion that GRP7 is a main target of HopU1 in reducing plant immunity, GRP7ox plants were as resistant to *Pto* DC3000 as WT plants to *Pto* DC3000  $\Delta$ *hopU1* (Supplementary Figure S2G). These results, together with previous results (Fu *et al*, 2007; Jeong *et al*, 2011), indicate that the abundance and/or activity of GRP7 are both required and limited for triggering optimal early and late PTI responses.

### **GRP7 associates with the immune receptors FLS2 and EFR at the plasma membrane**

The importance of GRP7 for early PTI responses suggests that GRP7 may affect directly PRRs and/or associated proteins, or indirectly the expression and/or biogenesis of such proteins.

Notably, we identified GRP7 in an unbiased yeast two-hybrid screen for EFR-interacting proteins (Supplementary Figure S3A). Importantly, we could confirm this interaction in co-immunoprecipitation experiments after transient co-expression of EFR and GRP7 as C-terminally tagged fusion proteins with HA and enhanced green fluorescent protein (GFP) tags (EFR-3 × HA and GRP7-eGFP, respectively) in *Nicotiana benthamiana* (Figure 2A). Similarly, GRP7 and FLS2 also interacted when transiently co-expressed as fusion proteins (FLS2-3 × myc and GRP7-eGFP) in *N. benthamiana* (Figure 2B). However, GRP7-eGFP did not interact under similar conditions with the LRR-RK BAK1 (BAK1-HA) (Figure 2C), which is an important positive regulator of PTI responses downstream of FLS2 and EFR (Chinchilla *et al*, 2007; Heese *et al*, 2007; Roux *et al*, 2011).

We confirmed the GRP7–FLS2 association by co-immunoprecipitation in an *A. thaliana* transgenic line expressing

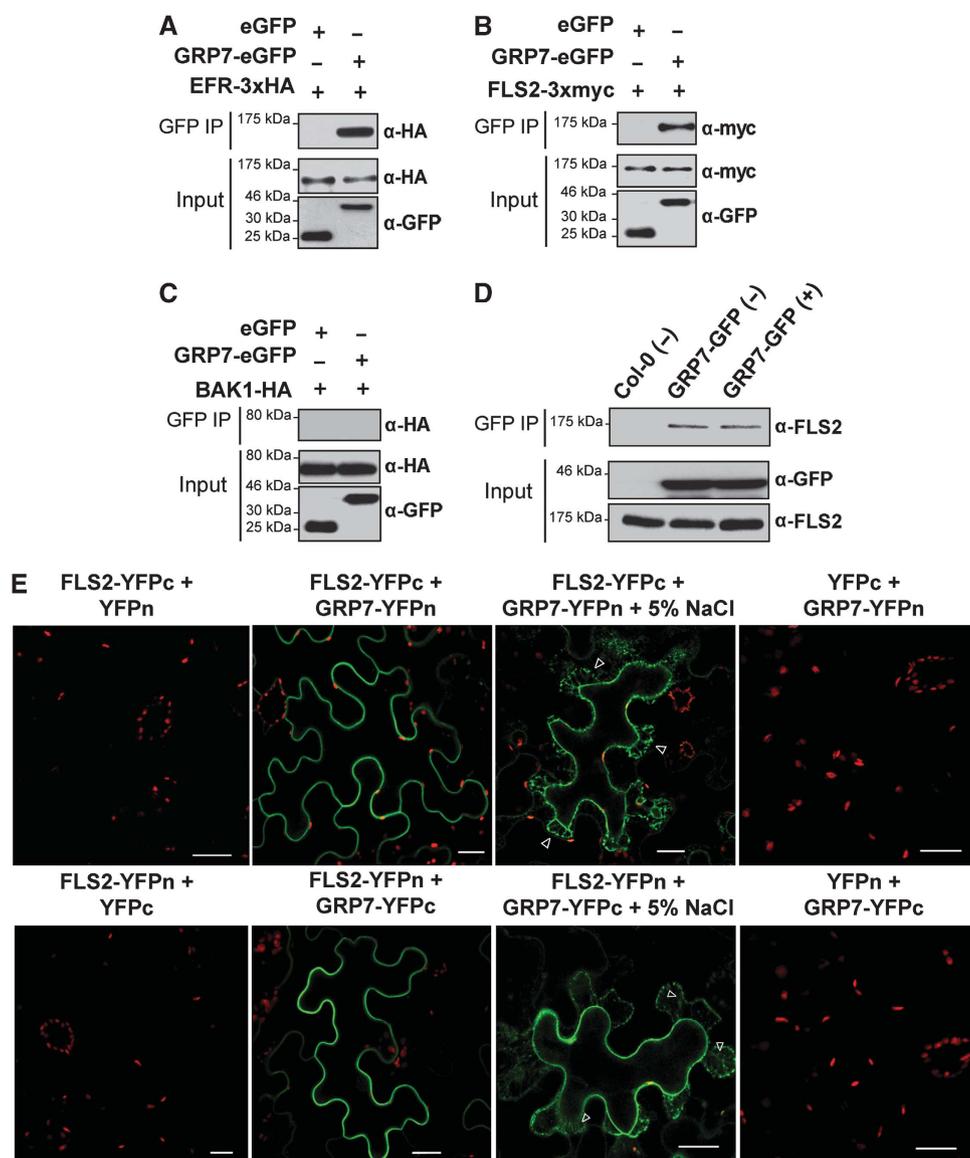


**Figure 1** GRP7 overexpression enhances significantly PTI responses and resistance to *Pseudomonas* infection. (A) Oxidative burst triggered by 1  $\mu$ M flg22, 1  $\mu$ M elf18, 100  $\mu$ g/ml chitin or in absence of PAMP treatment in Col-2 and transgenic *A. thaliana* plants overexpressing GRP7 (GRP7ox). ROS production is presented as total photon count during 25 min of treatment and measured in relative light units (RLUs). Values are mean  $\pm$  s.e. ( $n = 6$ ). Statistical significance was assessed using the ANOVA test ( $P < 0.001$ ). (B) Callose deposition induced by 1  $\mu$ M flg22, 1  $\mu$ M elf18, 100  $\mu$ g/ml chitin or in absence of PAMP treatment, directly infiltrated in Col-2 and transgenic *A. thaliana* plants overexpressing GRP7 (GRP7ox). Values are mean  $\pm$  s.e. ( $n = 24$ ). Statistical significance was assessed using the ANOVA test ( $P < 0.001$ ). ND, non-detectable. (C) Growth of *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 on Col-2 and GRP7ox plants as measured by colony forming units (cfu). Bacterial growth was measured 4 days after spray inoculation with the wild-type strain (WT) or the *hrcC*<sup>-</sup> strain. Values are mean  $\pm$  s.e. ( $n = 4$ ). dai, days after inoculation. Statistical significance was assessed using the ANOVA test ( $P < 0.001$ ; letters indicate statistically significant differences). (D) Disease symptoms on Col-2 and GRP7ox plants, 4 days after spray infection with *Pto* DC3000 WT. All results shown are representative of at least three independent experiments.

GRP7 C-terminally tagged with a GFP epitope (GRP7-GFP) under the control of the 35S promoter (Kim *et al*, 2008) and using an anti-FLS2 antibody recognizing the native FLS2 protein (Figure 2D). The GRP7-FLS2 interaction occurred independently of elicitation and was unaltered by flg22 treatment (Figure 2D). The presence of EFR and FLS2 proteins that may correspond to their glycosylated forms (migrating at  $\sim 150$  kDa and  $\sim 175$  kDa, respectively Nekrasov *et al*, 2009; Haweker *et al*, 2010) in the GRP7 immunoprecipitates (Figures 2A, B and D) suggests that the

association between GRP7 and PRRs occurs at the plasma membrane once the mature and functional PRRs have migrated through the secretory pathway.

GRP7-GFP shows a nucleo-cytoplasmic subcellular localization in *A. thaliana*, tobacco (*Nicotiana tabacum*) and *N. benthamiana* cells upon stable or transient expression (Supplementary Figures S4A and B; Ziemienowicz *et al*, 2003; Fu *et al*, 2007; Kim *et al*, 2008; Lummer *et al*, 2011). Bimolecular fluorescence complementation (BiFC) experiments using split-yellow fluorescent protein (YFP) following



**Figure 2** GRP7 associates with FLS2 at the plasma membrane. (A–C) Co-immunoprecipitation assay performed after transient co-expression of GRP7-eGFP or eGFP with EFR-3 × HA (A), FLS2-3 × myc (B) or BAK1-HA (C) in *N. benthamiana* plants. Total proteins (input) were subjected to immunoprecipitation with GFP Trap beads followed by immunoblot analysis. (D) Co-immunoprecipitation of GRP7 and FLS2 in *A. thaliana*. Co-immunoprecipitation assay performed on Col-0 and GRP7-GFP plants untreated (–) or treated (+) with 1 μM flg22 for 15 min. Total proteins (input) were subjected to immunoprecipitation with GFP Trap beads followed by immunoblot analysis. (E) Bimolecular fluorescence complementation assays between GRP7 and FLS2. YFPn, GRP7-YFPn, YFPc and FLS2-YFPc, as well as the reverse combinations YFPc, GRP7-YFPc, YFPn and FLS2-YFPn, were transiently co-expressed in *N. benthamiana* leaves. Plasmolysis experiment was performed in the presence of 5% NaCl for 5 min. Arrows indicate Hechtian strands. The chlorophyll autofluorescence appears in red. Scale bar corresponds to 20 μm. Photographs were taken 2 days after infiltration and are representative of the total observations (*n* = 60). All results shown are representative of three independent experiments.

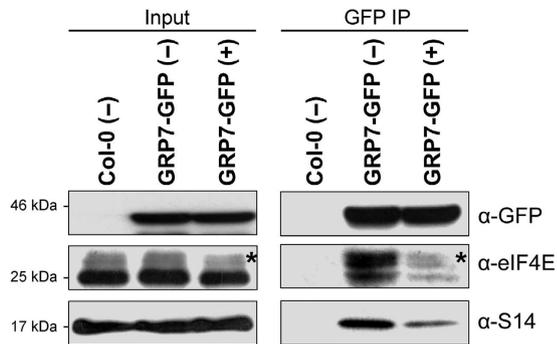
transient expression in *N. benthamiana* suggest that GRP7 and FLS2 closely associate (Figure 2E). This interaction occurs most likely at the plasma membrane, as indicated by the presence of the reconstituted YFP signal in typical cell wall–plasma membrane connections (called Hechtian strands) after cell plasmolysis (arrows in Figure 2E). An interaction at the cell periphery between GRP7 and EFR could also be observed (Supplementary Figure S3B).

#### GRP7 associates with translational components

In exploratory experiments to identify GRP7 interactors *in planta* by immunoprecipitation using an *A. thaliana* transgenic line expressing GRP7 C-terminally tagged with HA

under the control of its native promoter (GRP7-HA) (Jeong *et al*, 2011), we identified by mass-spectrometry analysis of the GRP7-HA immunoprecipitates several components of the 43S complex involved in protein translation (Supplementary Table S1). Before the initiation of active translation, the 43S complex recruits both mRNAs and ribosomes, and is composed of several initiation factors in addition to the cap-binding protein eIF4E and the ribosomal 40S subunit (Pestova *et al*, 2001).

Co-immunoprecipitation experiments using the *A. thaliana* GRP7-GFP transgenic line (Kim *et al*, 2008) and specific antibodies further revealed the presence of eIF4E and the ribosomal subunit S14 in complex with GRP7



**Figure 3** GRP7 associates with translational components in *Arabidopsis*. Co-immunoprecipitation of GRP7 and translational components in *A. thaliana*. Co-immunoprecipitation assay performed on Col-0 and GRP7-GFP plants untreated (-) or treated (+) with 1  $\mu$ M flg22 for 15 min. Total proteins (input) were subjected to immunoprecipitation with GFP Trap beads followed by immunoblot analysis with anti-GFP antibodies to detect GRP7-GFP or specific antibodies recognizing the translation initiation factor eIF4E and the ribosomal protein S14. Asterisks mark eIF4E slower migrating bands. The results shown are representative of three independent experiments.

(Figure 3). We used here the GRP7-GFP line for consistency with previous targeted co-immunoprecipitation experiments (Figure 2). Interestingly, slower migrating bands of eIF4E were enriched in GRP7-GFP immunoprecipitates in comparison to the main form detected in the input (see asterisks in Figure 3). Strikingly, treatment with flg22 induced the dissociation of eIF4E and S14 from the GRP7 complex (Figure 3), indicating a potential dynamic link between GRP7, ligand-activated PRRs and components of the translational machinery.

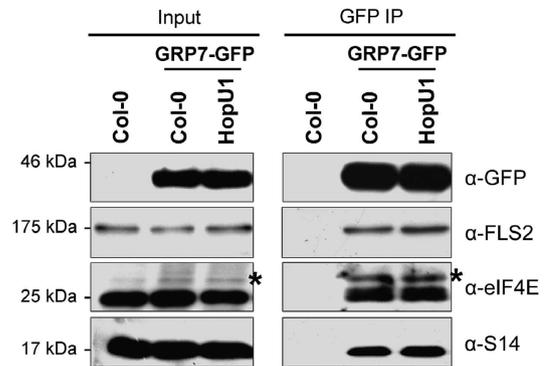
#### **HopU1 does not affect interactions between GRP7, PRRs and translational components**

Next, we tested if HopU1 could directly affect FLS2 or the GRP7-FLS2 interaction. HopU1 did not interact with FLS2 *in vivo* as determined by co-immunoprecipitation and split-YFP experiments in *N. benthamiana* (Supplementary Figures S5A and B). Consistently, HopU1 did not mono-ADP-ribosylate FLS2 *in vitro* (Supplementary Figure S5C).

Although HopU1 directly interacts with GRP7 *in vivo* (Supplementary Figure S6), HopU1 did not affect the interaction between GRP7 and FLS2 in an *A. thaliana* transgenic line expressing both GRP7-GFP and HopU1-HA (Figure 4). In addition, HopU1 did not interfere, at least qualitatively, with the association or ligand-induced dissociation between GRP7-GFP and either eIF4E or S14 (Supplementary Figure S7). Therefore, the effect of HopU1 on PTI responses is most likely not mediated by direct inhibition of PRRs or protein-protein interactions with GRP7.

#### **GRP7 associates with FLS2 and EFR transcripts in planta**

Next, we investigated the role of GRP7 in PTI in relation to its capacity to bind RNA by testing if GRP7 could bind PRR transcripts. Quantitative RNA-immunoprecipitation assays using the *A. thaliana* GRP7-HA (Jeong *et al*, 2011) transgenic line revealed that GRP7 binds *FLS2* mRNAs *in vivo* independently of flg22 treatment (Figure 5A). As positive controls, we confirmed that GRP7 binds its own transcripts as well as transcripts of its closest paralogue *GRP8* (Figure 5A),

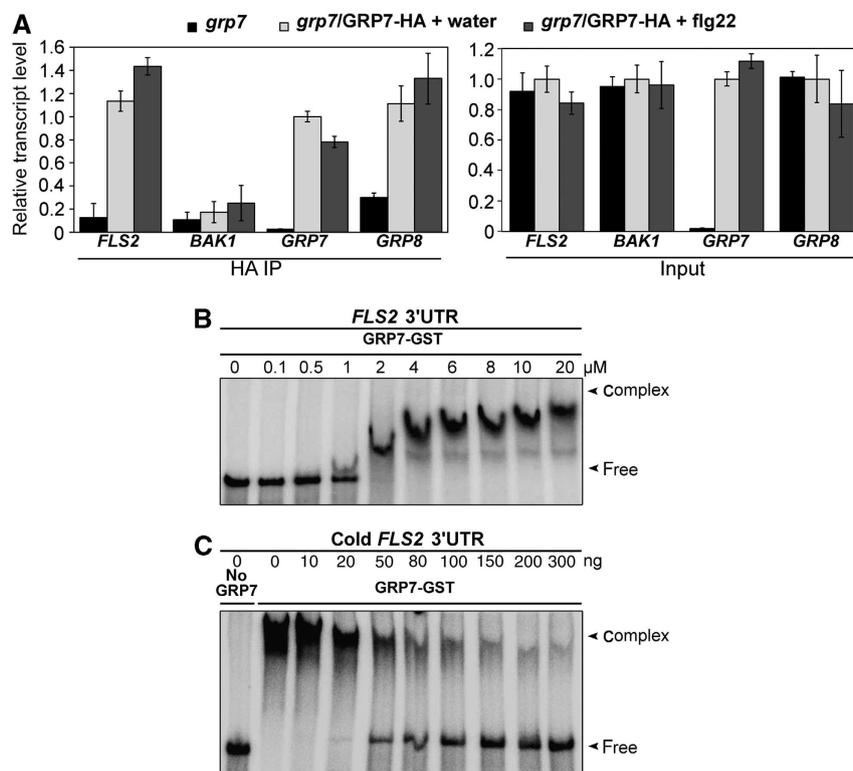


**Figure 4** HopU1 does not affect the protein-protein interactions between GRP7, FLS2 and translational components. Co-immunoprecipitation of GRP7-associated proteins in the presence of HopU1 in *A. thaliana*. Co-immunoprecipitation assay performed on Col-0 and HopU1 plants expressing or not GRP7-GFP. Total proteins (input) were subjected to immunoprecipitation with GFP Trap beads followed by immunoblot analysis with anti-GFP antibodies to detect GRP7 or specific antibodies recognizing FLS2, the translation initiation factor eIF4E, or the ribosomal protein S14. Asterisks mark slower migrating band forms. The results shown are representative of three independent experiments.

as previously reported *in vitro* (Staiger *et al*, 2003; Schoning *et al*, 2008). GRP7 binds the 3'-UTR of its own transcript and of the *GRP8* mRNA (Schoning *et al*, 2007). Similarly, we identified the 3'-UTR as a binding region of GRP7 in the *FLS2* mRNA (Figures 5B and C). In addition to the *FLS2* mRNA, GRP7 could also bind the *EFR* transcript *in vivo* (Supplementary Figure S8A), consistent with the importance of GRP7 for responses triggered by both flg22 and elf18 (Jeong *et al*, 2011; Figure 1). However, transcripts of the regulatory LRR-RK BAK1 were not enriched in GRP7 immunoprecipitates (Figure 5A; Supplementary Figure S8A), revealing a certain degree of specificity. Interestingly, GRP8, which is also targeted by HopU1 (Fu *et al*, 2007), is also able to bind *FLS2* and *EFR* mRNAs (Supplementary Figure S9). These results demonstrate that GRP7, as well as GRP8, bind transcripts of the important PRRs FLS2 and EFR.

#### **HopU1 disrupts the association between GRP7 and PRR transcripts**

Next, we investigated if HopU1 could affect the ability of GRP7 to bind its target mRNAs, including *FLS2* and *EFR* transcripts. Using an *A. thaliana* transgenic line expressing both GRP7-GFP and HopU1-HA in quantitative RNA-immunoprecipitation assays, we found that the amount of *FLS2* and *EFR* transcripts bound to GRP7-GFP was strongly reduced in the presence of HopU1 (Figure 6A; Supplementary Figure S8A). A similar effect was observed on the interaction between GRP7 and its own mRNA (Figure 6A; Supplementary Figure S8A). Furthermore, a GRP7(R49K) variant, which carries a mutation in a conserved arginine residue within the RRM RNA-binding domain that is mono-ADP-ribosylated by HopU1 (Jeong *et al*, 2011), is strongly impaired in its ability to bind *FLS2*, *EFR*, *GRP7* and *GRP8* transcripts (Figure 6B; Supplementary Figure S8B). Furthermore, ADP ribosylation of GRP7 by HopU1 (but not by the catalytically inactive HopU1DD variant; Fu *et al*, 2007; Jeong *et al*, 2011) completely blocks the binding of GRP7 to the 3'-UTR of *FLS2* mRNA *in vitro* (Figure 6C). Together, our results suggest that the mono-ADP ribosylation of GRP7 by



**Figure 5** GRP7 binds *FLS2* transcript. (A) RNA immunoprecipitation in *grp7-1* and *grp7-1/GRP7-HA* *A. thaliana* lines treated for 30 min with water or 1 μM flg22. Total proteins were subjected to immunoprecipitation with anti-HA antibodies followed by quantitative RT-PCR analysis of *FLS2*, *BAK1*, *GRP7* and *GRP8* transcripts with specific primers. Values are mean ± s.e. ( $n=4$ ). The results shown are representative of three independent experiments. (B, C) GRP7 binds the 3'UTR of *FLS2* transcripts *in vitro*. Electrophoretic shift assays performed on the 3'UTR of *FLS2* RNAs, in presence of increasing concentrations of GRP7-GST (B). Competition assay was performed with increasing quantity of unlabelled *FLS2* 3'UTR transcripts to GRP7-GST and  $^{32}$ P-labelled *FLS2* 3'UTR transcripts (C). The results shown are representative of three independent experiments.

HopU1 disrupts *in planta* the ability of GRP7 to bind mRNAs of the PRRs *FLS2* and *EFR*.

### HopU1 inhibits the pathogen-induced *FLS2* protein accumulation during *Pseudomonas* infection

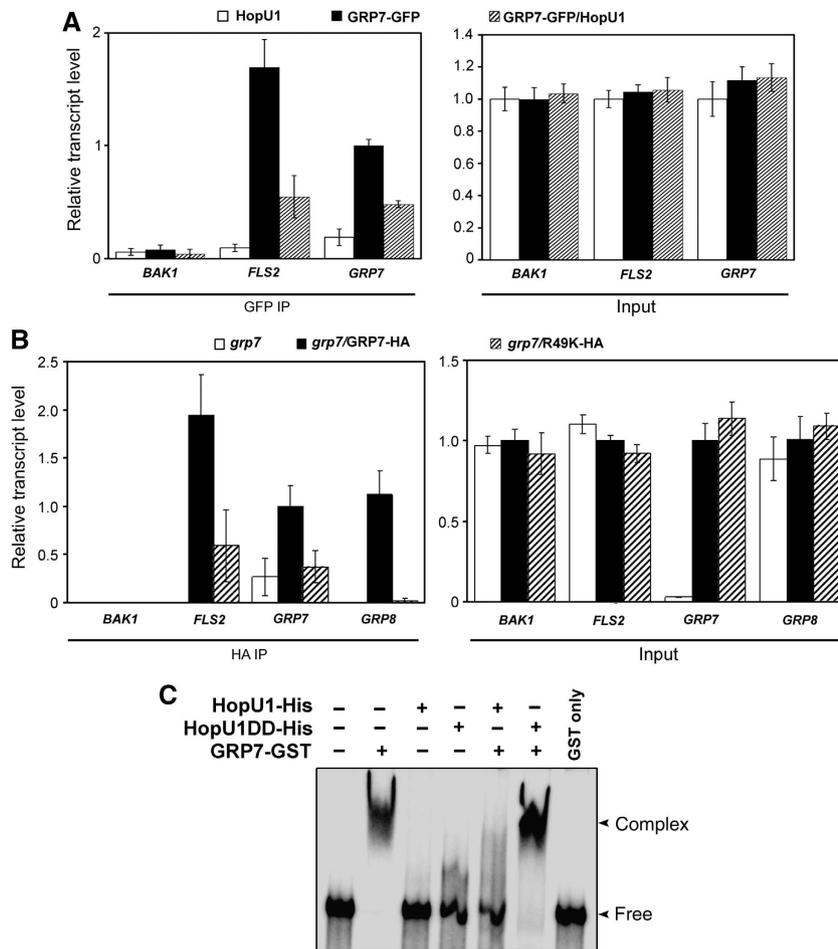
Because HopU1 inhibits GRP7-*FLS2* mRNA binding (Figure 6), we asked whether HopU1's action could ultimately affect *FLS2* protein levels after translocation into *A. thaliana* cells during *Pto* DC3000 infection, which would correspond to the most biologically relevant observation. We observed that the amount of *FLS2* protein increases (3.5- to 3.8-fold) over 24 h in leaves infected with *Pto* DC3000 *hrcC*<sup>-</sup> (unable to secrete any T3SEs and therefore unable to dampen PTI) (Figure 7A), consistent with the previous observation that the expression of *FLS2*, *EFR* and other potential PRR-encoding genes is PAMP inducible (Zipfel et al, 2004, 2006). Notably, this PAMP-induced accumulation is attenuated by T3SEs (Figure 7A; compare *hrcC*<sup>-</sup> with WT). However, this T3SE-mediated suppression was much less marked after inoculation with *Pto* DC3000  $\Delta$ *hopU1* (Figure 7A; compare  $\Delta$ *hopU1* with WT). Importantly, expression of *HopU1* *in trans* on a plasmid in *Pto* DC3000  $\Delta$ *hopU1* restored the inhibition of *FLS2* accumulation during infection, while trans-complementation with the catalytically inactive HopU1DD variant (Fu et al, 2007; Jeong et al, 2011) did not (Figure 7B). In addition, HopU1 does not affect *BAK1* levels during infection (Supplementary Figure S10); consistent with our previous

finding that GRP7 does not bind *BAK1* mRNA (Figures 5 and 6; Supplementary Figure S8).

Notably, while the amount of cellular *FLS2* mRNA increased during the first hours of infection with *Pto* DC3000 *hrcC*<sup>-</sup>, it decreased to a similar level upon infection with *Pto* DC3000 WT and  $\Delta$ *hopU1* (Figure 7C). The contrasting regulation and sensitivity to HopU1 of *FLS2* mRNA and protein levels during infection further demonstrate that HopU1 regulates *FLS2* post-transcriptionally, while other T3SEs already regulate *FLS2* at the transcriptional level. Consistent with *FLS2* being an important virulence target for HopU1, we found that deletion of the flagellin-encoding gene *fliC* in the  $\Delta$ *hopU1* background (*Pto* DC3000  $\Delta$ *hopU1*  $\Delta$ *fliC*) suppresses the virulence defect of *Pto* DC3000  $\Delta$ *hopU1* and restores the virulence of *Pto* DC3000  $\Delta$ *fliC* on WT *A. thaliana* plants (Figure 7D). Together, these results indicate that HopU1 strongly affects the increased accumulation of *FLS2* protein level normally observed during the first hours of *A. thaliana* infection by *Pto* DC3000.

## Discussion

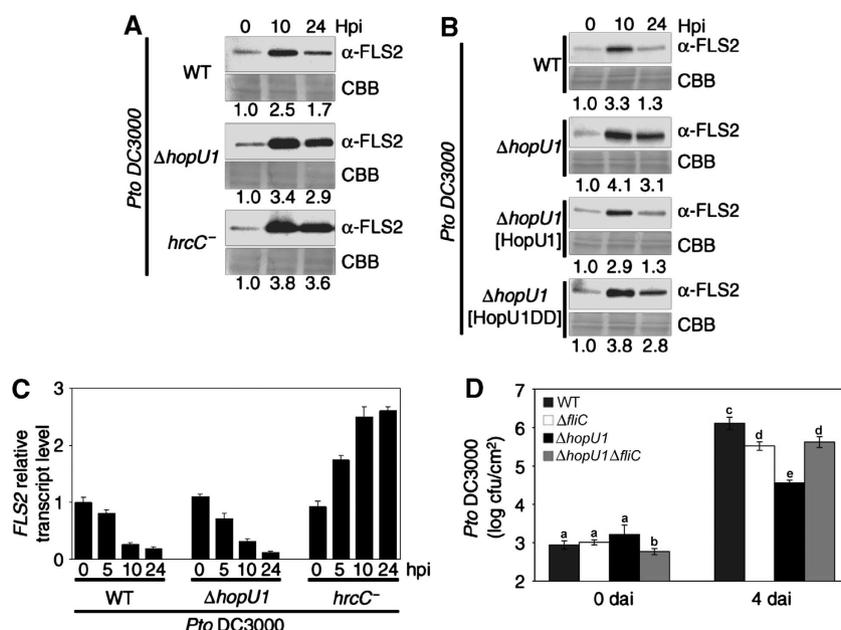
Several animal and plant pathogenic bacteria use toxins or T3SEs that possess mono-ADP-RT activity as major virulence factors via the targeting of distinct host proteins (Deng and Barbieri, 2008; Block and Alfano, 2011). The mono-ADP-RT toxins diphtheria toxin (DT) from *Corynebacterium*



**Figure 6** HopU1 disrupts GRP7-*FLS2* transcripts interactions. (A) RNA immunoprecipitation in HopU1, GRP7-GFP and GRP7-GFP/HopU1 *A. thaliana* lines. Total proteins were subjected to immunoprecipitation with GFP Trap beads followed by quantitative RT-PCR analysis of *BAK1*, *FLS2* and *GRP7* transcripts with specific primers. Values are mean  $\pm$  s.e. ( $n = 4$ ). (B) RNA immunoprecipitation in *grp7*, *grp7/GRP7-HA* and *grp7/GRP7(R49K)-HA* *A. thaliana* lines. Total proteins were subjected to immunoprecipitation with anti-HA matrix beads followed by quantitative RT-PCR analysis of *BAK1*, *FLS2*, *GRP7* and *GRP8* transcripts with specific primers. Values are mean  $\pm$  s.e. ( $n = 4$ ). (C) Electrophoretic shift assays in the presence of HopU1 and its inactive version HopU1DD. Standard ADP-ribosylation reaction was performed with 4  $\mu$ M GRP7-GST in the presence of 1  $\mu$ M HopU1 or HopU1DD. The corresponding GRP7-GST was then added to the 3'-UTR of *FLS2* transcript binding assay. The results shown are representative of three independent experiments.

*diphtheriae*, ExoA from *P. aeruginosa* and cholix toxin (CT) from *Vibrio cholerae* interact with the eukaryotic elongation factor eEF2 as a unique specific host target (Deng and Barbieri, 2008; Jorgensen *et al*, 2008). In contrast, other mono-ADP-RTs (e.g., the T3SE ExoS from *P. aeruginosa*) target numerous host proteins (Hauser, 2009). The study of mono-ADP-RT T3SEs from phytopathogenic bacteria is still in its infancy. HopU1 from *Pto* DC3000 was the first from this family with a proven role in virulence and targets several plant RBPs, including GRP7 and GRP8 (Fu *et al*, 2007). More recently, the plasma membrane-targeted T3SE mono-ADP-RT HopF2 from *Pto* DC3000 was also shown to target multiple plant proteins, including the MAP kinase kinase MKK5 and the important immune regulator RIN4, to suppress PTI and cause disease (Wang *et al*, 2010; Wilton *et al*, 2010; Wu *et al*, 2011). The T3SE HopF1/AvrPphF from *P. syringae* pv. *phaseolicola* race 7 shows weak homology to mono-ADP-RTs and is required for virulence on bean (Tsiamis *et al*, 2000; Singer *et al*, 2004), but no corresponding host target has been identified so far.

GRP7 is required for PTI responses triggered by several PAMPs, including flg22, elf18 and chitin (Fu *et al*, 2007; Jeong *et al*, 2011). In addition, we have shown that GRP7 overexpression increases early and late PTI responses triggered by these PAMPs (Figure 1). Similarly, loss of and overexpression of GRP7 leads to reduced and increased resistance to *Pto* DC3000, respectively (Figure 1; Fu *et al*, 2007; Jeong *et al*, 2011). These results demonstrate that GRP7 is an important immune regulator that is both required and limiting for full immunity to *Pto* DC3000. Despite these findings, the exact role of GRP7 in PTI was still unclear. RBPs can be involved in different aspects of RNA metabolism (e.g., splicing, transport, storage, translation and degradation) (Keene, 2007). According to a potential role in RNA-related processes, we found that GRP7 associates *in vivo* specifically with several components of the translational machinery, including eIF4A1, eIF4A2, eEF1A, eIF4E and S14 (Figures 3 and 4; Supplementary Figure S7; Supplementary Table S1). The association of GRP7 with an active translational complex is further suggested by the enrichment of a



**Figure 7** HopU1 inhibits FLS2 protein accumulation during infection. (A) Immunoblots with specific antibodies detecting endogenous FLS2 in Col-0 during bacterial infection after syringe inoculation with *Pto* DC3000 (WT; inoculum:  $5 \times 10^7$  cfu/ml), *Pto* DC3000  $\Delta hopU1$  (inoculum:  $10^8$  cfu/ml), *Pto* DC3000  $hrcC^-$  (inoculum:  $10^8$  cfu/ml). hpi, hours post infection; CBB, Coomassie Brilliant Blue. Values correspond to signal intensity of the FLS2-specific band from the immunoblots relative to the zero time point. (B) Immunoblots with specific antibodies detecting endogenous FLS2 in Col-0 during bacterial infection after syringe inoculation with *Pto* DC3000 (WT; inoculum:  $5 \times 10^7$  cfu/ml), *Pto* DC3000  $\Delta hopU1$  (inoculum:  $10^8$  cfu/ml), *Pto* DC3000  $\Delta hopU1$  [HopU1] (inoculum:  $10^8$  cfu/ml), *Pto* DC3000  $\Delta hopU1$  [HopU1DD] (inoculum:  $10^8$  cfu/ml). hpi, hours post infection; CBB, Coomassie Brilliant Blue. Values correspond to signal intensity of the FLS2-specific band from the immunoblots relative to the zero time point. (C) FLS2 transcript level as measured by quantitative RT-PCR in Col-0 plants during bacterial infection after syringe inoculation with *Pto* DC3000 (WT; inoculum:  $5 \times 10^7$  cfu/ml), *Pto* DC3000  $\Delta hopU1$  (inoculum:  $10^8$  cfu/ml), *Pto* DC3000  $hrcC^-$  (inoculum:  $10^8$  cfu/ml). hpi, hours post infection. (D) Bacterial growth measured during *Pseudomonas* infection in Col-0 plants, after spray inoculation (inoculum:  $2 \times 10^8$  cfu/ml) with *Pto* DC3000 wild-type (WT) or the derived strains  $\Delta fliC$ ,  $\Delta hopU1$  and  $\Delta hopU1 \Delta fliC$ . Growth measured by colony forming units (cfu) 4 days after inoculation. Values are mean  $\pm$  s.e. ( $n = 4$ ). Statistical significance was assessed using the ANOVA test ( $P < 0.001$ ; letters indicate statistically significant differences). dai, days after inoculation. The results shown are representative of three independent experiments.

slower migrating form of eIF4E in the GRP7 immunoprecipitate (Figures 3 and 4; Supplementary Figure S7), which may correspond to the phosphorylated active form of eIF4E that is linked to the mRNA cap (Beretta *et al*, 1998; Raught and Gingras, 1999; Dyer *et al*, 2003).

Unexpectedly, we also found that GRP7 directly interacts *in vivo* with the PRRs FLS2 and EFR in a specific manner (Figure 2; Supplementary Figure S3), as GRP7 did not interact with the LRR-RLK BAK1 (Figure 2C) that is involved in PTI signalling downstream of FLS2 and EFR (Chinchilla *et al*, 2007; Heese *et al*, 2007; Roux *et al*, 2011). Notably, the interaction between GRP7 and FLS2 (and EFR) appears to be at the plasma membrane, as indicated by the localization of the YFP signal in BiFC experiments (Figure 2E; Supplementary Figure S3B) and the presence of FLS2 (and EFR) in co-immunoprecipitation experiments (Figures 2A, B and D). In addition, GRP7 and GRP8 are present in highly purified *A. thaliana* plasma membrane preparations (Alexandersson *et al*, 2004). Interestingly, a potential direct and dynamic link between the GRP7-FLS2 complex and the complex between GRP7 and translational components is indicated by the flg22-induced dissociation of eIF4E and S14 from the GRP7 complex (Figure 3). This observation is reminiscent of the recent demonstration in mammals and plants of transmembrane receptor-associated translational regulation complexes that most likely enable rapid activation of ligand-induced translation (Ehsan *et al*, 2005; Carvalho *et al*, 2008; Lin *et al*, 2010; Tcherkezian *et al*, 2010). Previous

electron microscopy experiments revealed the presence of polysomes anchored to the plasma membrane via actin filaments in both plant and animal cells (Hovland *et al*, 1996; Medalia *et al*, 2002). Therefore, the reported association of FLS2 with translation regulation complexes at the plasma membrane is of great interest for further studies.

However, we found that HopU1 does not directly target FLS2 by mono-ADP-ribosylation (Supplementary Figure S5), nor perturb the association or the dissociation between GRP7 and FLS2 or translational components (Figure 4; Supplementary Figure S7) despite directly interacting with GRP7 (Supplementary Figure S6). These results suggest that HopU1 targets another molecular function of GRP7 that is independent of the aforementioned protein-protein interactions. In addition to the previously reported binding of GRP7 to its own transcript and that of GRP8 *in vitro* (Staiger *et al*, 2003; Schoning *et al*, 2008), we found that GRP7 binds the transcripts of FLS2 and EFR *in vivo*, but not of BAK1 (Figure 5A; Supplementary Figure S8). As shown for its own transcript (Schoning *et al*, 2007), GRP7 binds the 3'-UTR of FLS2 mRNA *in vitro* (Figures 5B and C). To our knowledge, FLS2 and EFR correspond to the first described non-RBP cargo mRNAs bound by GRP7 and GRP8 with a clear biological function. However, it is clear that GRP7 carries other cargo mRNAs involved in immunity and other processes that require identification, as GRP7 has been also involved in circadian clock, flowering time and tolerance to abiotic stresses (Mangeon *et al*, 2010). An effect on these

cargo mRNAs may explain some of the immune phenotypes observed in the GRP7 overexpressing lines. Importantly, however, we demonstrated that HopU1 inhibits the GRP7-*FLS2* mRNA and GRP7-*EFR* mRNA interactions *in vivo*, and that these interactions depend on R49 (Figure 6; Supplementary Figure S8), the conserved residue that is mono-ADP-ribosylated by HopU1 and located within the RRM RNA-binding domain of GRP7 (Jeong *et al*, 2011). This is consistent with the previously reported importance of this residue for RNA binding *in vitro* (Schoning *et al*, 2007; Jeong *et al*, 2011). Our results provide examples of relevant immune-related target mRNAs whose binding to GRP7 is inhibited by HopU1 *in vivo*. Furthermore, we could show that the injection of HopU1 by the type III secretion system during *Pto* DC3000 infection leads to reduced *FLS2* accumulation *in planta* (Figure 7). Notably, the effect of HopU1 on *FLS2* amounts is comparable to the previously described effect of the *Pto* DC3000 T3SE AvrPtoB or the *Arabidopsis* E3 ligases PUB12/13 (Gohre *et al*, 2008; Lu *et al*, 2011). This suggests that the inability of GRP7 to bind *FLS2* mRNA ultimately results in decreased *FLS2* accumulation during infection, potentially by inhibiting *FLS2* translation.

In apparent contradiction to our model, we only found slightly reduced *FLS2* protein levels in HopU1 *A. thaliana* seedlings under sterile conditions (Supplementary Figure S2C). However, we hypothesize that GRP7 is not involved in steady-state synthesis of *FLS2*, but rather regulates pathogen-induced *FLS2* translation. Indeed, *FLS2* gene expression is induced upon PAMP treatment (Zipfel *et al*, 2004, 2006), and *FLS2* accumulates strongly upon infection with *Pto* DC3000 *hrcC*<sup>-</sup> (Figure 7A) that only triggers PTI, given its inability to secrete PTI-suppressing T3SEs. Notably, consistent with the fact that GRP7 does not bind *BAK1* mRNA (Figures 5 and 6; Supplementary Figure S8), HopU1 does not affect *BAK1* protein levels during infection (Supplementary Figure S10).

In accordance with our hypothesis, we observed that GRP7ox seedlings accumulate about 20% more *FLS2* protein after 4 h of flg22 treatment than WT seedlings (Supplementary Figure S11). Although we could not prove that *FLS2* mRNA was associated with GRP7 in complex with *FLS2* at the plasma membrane, our results suggest the existence of a complex including *FLS2* mRNA, GRP7, translational components and *FLS2* in a plasma membrane-associated complex that may contribute to pathogen-induced *de novo* translation of *FLS2* during the early steps of plant infection. An additional argument in favour of this hypothesis is that flg22 perception induces the release of the 43S complex from GRP7, potentially to start *de novo* protein synthesis. Notably, flg22 binding leads to *FLS2* endocytosis and *FLS2* degradation upon binding to E3 ligases (Robatzek *et al*, 2006; Lu *et al*, 2011). Therefore, it is possible that *de novo* pathogen-induced *FLS2* translation is involved in the rapid replenishment of the plasma-membrane pool of ligand-free *FLS2*. In this model, HopU1 would inhibit GRP7-*FLS2* mRNA interaction and thus block the recruitment of *FLS2* transcripts into the plasma membrane-associated complex, leading most likely to the inhibition of *de novo* pathogen-induced *FLS2* translation. Further work will be needed to test this model.

During the infection process, pathogenic effectors must collaborate in a complementary manner to suppress efficiently host innate immune responses. Importantly, the

secretion and action of T3SEs occur after or while PAMPs are perceived, given that the T3SS is only induced *in planta* (Boch *et al*, 2002). The T3SEs AvrPto and AvrPtoB target the *FLS2* pathway by blocking the activation of *FLS2* and/or *BAK1*, and by inducing *FLS2* degradation (Xing *et al*, 2007; Gohre *et al*, 2008; Shan *et al*, 2008; Xiang *et al*, 2008, 2011; Gimenez-Ibanez *et al*, 2009; Cheng *et al*, 2011; Zeng *et al*, 2012). In addition, the T3SEs AvrPphB and AvrAC target the immediate substrate of the *FLS2*-*BAK1* complex, *BKI1* and related proteins, for degradation or inhibition, respectively (Zhang *et al*, 2010; Feng *et al*, 2012). In this paper, we reveal that HopU1 may have a synergic effect with previously described *Pseudomonas* T3SEs by inhibiting PAMP-induced *de novo* *FLS2* synthesis, which would otherwise compensate for the negative action of AvrPto, AvrPtoB and AvrPphB on innate immunity. The importance of *FLS2* as an important virulence target for HopU1 is demonstrated by the strict dependency on the presence of flagellin to observe the hypo-virulence phenotype of *Pto* DC3000  $\Delta$ *hopU1* on WT *A. thaliana* plants (Figure 7D).

The demonstration that GRP7 binds PRR-encoding transcripts, and that the *Pto* DC3000 T3SE HopU1 inhibits this interaction in a manner dependent on its mono-ADP-RT activity ultimately leading to reduced PRR accumulation during infection, provides a first direct link between GRP7 and a function in PTI, and reveals a novel virulence mechanism employed by bacterial pathogens. This mechanism is in contrast with the virulence strategy employed by *C. diphtheriae*, *P. aeruginosa* and *V. cholerae* that use their mono-ADP-RT toxins (DT, ExoA and colix toxin, respectively) to eradicate host translation via the targeting of eEF2 (Deng and Barbieri, 2008; Jorgensen *et al*, 2008). In addition, the virulence strategy used by HopU1 differs from strategies deployed by previously described T3SEs from phytopathogenic bacteria that target the PRR proteins themselves or the signalling triggered by PRR activation (Block and Alfano, 2011).

## Materials and methods

### Plant material

*A. thaliana*, *N. benthamiana* and *N. tabacum* were grown at 20–21°C with a 10-h photoperiod in environmentally controlled chambers. *Arabidopsis* seedlings were grown on plates containing Murashige and Skoog (MS) medium (Duchefa) and 1% sucrose at 22°C with a 16-h photoperiod.

All experiments were performed in Col-0 background, except if indicated otherwise. The *fls2* mutant used in this study is SALK\_093905. The GRP7ox (Col-2/35S::GRP7), HopU1 (Col-0/35S::HopU1-HA), GRP7-GFP (Col-0/35S::GRP7-GFP), GRP7-HA (*grp7-1/GRP7p::GRP7-HA*) and GRP7(R49K)-HA [*grp7-1/GRP7p::GRP7(R49K)-HA*] were previously published in Fu *et al* (2007); Kim *et al* (2008); Streitner *et al* (2008); Jeong *et al* (2011). The GRP7-GFP/HopU1 line was obtained by crossing the GRP7-GFP and HopU1 lines.

The estradiol-inducible HopU1 line (ind\_HopU1) was obtained by transforming Col-0 with the vector pLN604 (derived from pER8; Zuo *et al*, 2000) carrying *hopU1-HA*. The expression of HopU1 was induced by spraying 15  $\mu$ M  $\beta$ -estradiol for 16–20 h. The GRP8-HA (Col-0/35S::GRP8-HA) line was obtained by transforming Col-0 with the binary vector pPZP212 carrying *GRP8-HA*. Homozygous lines with a single transgene insertion were used for the experiments.

### PTI assays

PAMP treatments (flg22 and elf18 peptides synthesized by Pepton, South Korea; shrimp chitin from SIGMA) were performed by syringe infiltration of plant leaves or by addition of the elicitor into the liquid media. Oxidative burst assays were performed on leaf disks

incubated in a solution containing luminol and peroxidase as previously described (Zipfel *et al*, 2004). Callose deposition was observed after infiltration with a solution of 1  $\mu$ M PAMP for 16 h as previously described (Hann and Rathjen, 2007). Callose deposits were quantified using PDQuest software (Bio-Rad).

### *Pseudomonas* infection

Bacterial strains used in this study were *Pseudomonas syringae* pv. *tomato* (Pto) DC3000 WT, Pto DC3000  $\Delta$ hopU1 and Pto DC3000 *hrcC*<sup>-</sup>. For bacterial enumeration assays, plants were sprayed with the strains WT (inoculum: 10<sup>6</sup> cfu/ml),  $\Delta$ hopU1 (inoculum: 10<sup>8</sup> cfu/ml) and *hrcC*<sup>-</sup> (inoculum: 10<sup>8</sup> cfu/ml), in the presence of 0.001% (v/v) Silwet L-77. Sprayed plants were then covered with a transparent plastic lid for the remaining of the experiment. For the other assays, bacteria were infiltrated into *Arabidopsis* leaves with the strains WT (inoculum: 5  $\times$  10<sup>7</sup> cfu/ml),  $\Delta$ hopU1 (inoculum: 10<sup>8</sup> cfu/ml) and *hrcC*<sup>-</sup> (inoculum: 10<sup>8</sup> cfu/ml).

For the trans-complementation experiment described in Figure 7, pLN1981, encoding HopU1-HA, and pLN1982, encoding HopU1DD-HA, were electroporated into UNL141, the DC3000  $\Delta$ hopU1 mutant (Fu *et al*, 2007). The  $\Delta$ hopU1(HopU1) and  $\Delta$ hopU1(HopU1DD) strains were confirmed to produce HopU1-HA and HopU1DD-HA prior to using these strains to infect *Arabidopsis*.

The construction of  $\Delta$ fliC mutant was done by unmarked mutagenesis (House *et al*, 2004). A 1.9-kb upstream (US) DNA sequence and 1.5-kb downstream (DS) were amplified by PCR using the primers fliC us-F (CACCGAGTTTACATGCAACGCCTG) and fliC us-R (CATGATGAATTCCTCGTTGG) and fliC ds-F (CACCCAGTAATATCGGCATGAG) and fliC-R (CGCTGATCGAACCCTTGCTC). The purified PCR products were cloned into the pENTR/D-TOPO vector (Invitrogen). Entry constructs were then recombined by LR reactions into pMK2017 for US sequence and pMK2016 for DS sequences. These plasmids were separately introduced into DC3000 and plated onto media selecting for integration of each plasmid into DC3000 genome. Deletion of *fliC* was performed following a previously published protocol (Crabill *et al*, 2010). For construction of the *fliC* hopU1 double mutant,  $\Delta$ fliC was used as a background strain to generate a *hopU1* deletion mutation via homologous recombination using constructs described in Fu *et al* (2007). The resulting mutants were confirmed with PCR using primers that annealed to the flanking regions of either *fliC* or *hopU1*.

### Yeast two-hybrid

The coding region corresponding to the cytoplasmic part of EFR (EFR<sub>cyt</sub>) was cloned in the pLexA vector. The recombinant pLexA-EFR<sub>cyt</sub> was used to screen a cDNA library prepared from infected *Arabidopsis* plants (van der Biezen *et al*, 2000) according to the indications of the manufacturer (LexA system-based yeast two hybrid; CLONTECH). The coding region of GRP7 from the nucleotides 67 to 528 (corresponding to the region of the GRP7 clone identified during the initial screen) was re-cloned in the pB42AD vector to retest the interaction.

### *Agrobacterium*-mediated transient expression for co-immunoprecipitation, subcellular localization and BiFC experiments

For the co-immunoprecipitation and subcellular localization experiments, we used the following previously described constructs: 35S::GRP7-eGFP (Fu *et al*, 2007), 35S::EFR-3  $\times$  HA (Schwessinger *et al*, 2011), 35S::FLS2-3  $\times$  myc (Chinchilla *et al*, 2006), 35S::BAK1-HA (Schwessinger *et al*, 2011), 35S::FLS2-GFP-His (Schwessinger *et al*, 2011) and 35S::HopU1-HA (Fu *et al*, 2007). The *Agrobacterium* strains GV3101 carrying the indicated constructs were syringe-infiltrated in *N. benthamiana* or *N. tabacum* leaves at OD<sub>600</sub> = 0.4–0.6 and samples were collected 2 days post infiltration.

For the BiFC assays, we cloned the coding regions of *FLS2*, *EFR*, *GRP7* and *HopU1* into the BiFC binary vectors pAM-PAT-35S that were previously described in Lefebvre *et al* (2010). The *Agrobacterium* strain GV3101 expressing the silencing suppressor P19 (Voinnet *et al*, 2003) and carrying the indicated constructs were syringe-infiltrated in *N. benthamiana* leaves at OD<sub>600</sub> = 0.4–0.6.

Confocal analyses for the subcellular localization and BiFC experiments were performed 2 days post infiltration using a Leica SP5 confocal microscope.

### Protein extraction, co-immunoprecipitation and immunoblotting

Total proteins were extracted in a buffer including 100 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 5% glycerol, 10 mM DTT, 0.5% Triton X-100, 1% Igepal and protease inhibitors (Sigma). For co-immunoprecipitation, anti-HA beads (Roche) or anti-GFP-TRAP-A beads (Chromotek) were incubated with total proteins and then washed with the extraction buffer. Proteins were fractionated on SDS-PAGE, transferred onto PVDF membranes (Bio-Rad) and then detected using specific antibodies. FLS2 was detected using specific polyclonal antibodies raised in rabbit as primary antisera. The anti-S14-1 antibodies were purchased (Agrisera, Sweden). The rabbit anti-eIF4E antibody was a kind gift from Professor A Maule (John Innes Centre, Norwich, UK). Epitope-tagged proteins were detected with a peroxidase-conjugated anti-HA-HRP (Santa Cruz); mouse monoclonal anti-GFP antibodies (AMS); or anti-HRP antibodies (Santa Cruz). The secondary anti-rabbit-HRP antibodies (Sigma) were used when appropriate. Immunodetection was performed with ECL chemiluminescence reagent (GE). Tandem mass-spectrometry experiments have been performed as previously described (Fu *et al*, 2007).

### In vitro ADP-ribosylation assay

HopU1-His, GRP7-GST and FLS2-GST were affinity-purified from *Escherichia coli* BL21 and the purity of the proteins was examined by SDS-PAGE. The ADP-ribosylation assay was performed as previously (Fu *et al*, 2007).

### RNA-immunoprecipitation assay

After UV-crosslinking treatments (120 MJ, 3 times using UV Stratalinker<sup>TM</sup> 2400, Stratagene), total proteins were extracted in extraction buffer including 50 mM Tris-HCl pH 8, 150 mM NaCl, 2.5 mM EDTA, 10% glycerol, 10 mM PMSF, 10 units/ml RNaseOUT (Invitrogen) and protease inhibitors (Sigma). After centrifugation, the supernatant was incubated with anti-HA affinity matrix (Roche) or anti-GFP-TRAP-A beads (Chromotek). After this incubation, the beads were washed and the RNA-GRP7 complexes were eluted by incubating at 60°C for 15 min in the elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>). A proteinase K treatment for 1 h at 60°C was then followed by RNA extraction and quantitative RT-PCR.

In each experiment, transcript levels from the input have been normalized in comparison to the control samples (*grp7*/GRP7-HA in Figures 5A and 6B and Supplementary Figure S7B; HopU1 in Figure 6A and Supplementary Figure S7A; and Col-0 in Supplementary Figure S8). The normalization of the transcript levels after RNA-IP has been performed in comparison to the *GRP7* transcript level in *grp7*/GRP7-HA or GRP7-GFP samples (Figures 5A, 6A and B, Supplementary Figures S7A and B) or the *GRP8* transcript level (Supplementary Figure S8).

### Quantitative RT-PCR

After RNA extraction (Tri Reagent, Sigma-Aldrich), first-strand cDNA was synthesized using the SuperScript II Reverse Transcriptase (Invitrogen). Quantitative PCRs were performed from 1.5  $\mu$ l of cDNA with SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) on a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA). Primers used are

**Ubox** (At5g15400): 5'-TGCGCTGCCAGATAATACACTATF-3' and 5'-TGCTGCCCAACATCAGGTT-3'  
**FLS2** (At5g46330): 5'-ACTCTCTCCAGGGGCTAAGGAT-3' and 5'-AGCTAACAGCTCTCCAGGGATGG-3'  
**EFR** (At5g20480): 5'-CGGATGAAGCAGTACGAGAA-3' and 5'-CCATTCCTGAGGAGAAGTTTG-3'  
**BAK1** (At4g33430): 5'-ACCGCTCTATCTCTCTTACACC-3' and 5'-CTGGTCTCTTTCAGCTGGTACA-3'  
**GRP7** (At2g21660): 5'-TGATGACAGAGCTTTGAGACTGCC-3' and 5'-TCCTCTCCACCCTCGGCTTACCGCCGCCA-3'  
**GRP8** (At4g39260): 5'-CAATGATGAAGATCTTCAAAGGACG-3' and 5'-CTCGTAACCACCACCGCTCTCTGAGTATCC-3'

### Electrophoretic mobility shift assay

The 3'-UTR sequence of *FLS2* was amplified with the upstream primer GATGGTACCGAAGTTTAGCAGCAAAGC and the downstream primer GAGCTCGAGGTTTCATCAAACCAAATTTTC. The amplified fragment was subcloned into the plasmid pBSK(-) (Stratagene) and transcribed with T7 polymerase (Promega) in the presence of 10  $\mu$ Ci <sup>32</sup>P CTP.

The *FLS2* 3'-UTR binding affinity was analysed with purified GRP7-GST in 20 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.01% NP-40, 10 U SUPERase•In (Ambion), and 50 ng of <sup>32</sup>P-labelled *FLS2* 3'-UTR. Inhibition of RNA binding ability by HopU1 was tested by first producing mono-ADP-ribosylated GRP7, then performing an electrophoretic mobility shift assay. Four μM GRP7-GST was incubated with 1 μM HopU1-His or HopU1DD-His in the standard ADP-ribosylation reaction followed by analysis of RNA binding ability. For competition assays, unlabelled *FLS2* 3'-UTR transcripts were added into the mixture of 2 μM GRP7-GST and 50 ng of <sup>32</sup>P-labelled *FLS2* 3'-UTR transcripts. The bound and free RNA probes were separated on 6% native PAGE and exposed to PhosphorImager screen, then analysed by Storm 860 scanner (Molecular Dynamics).

### Statistical analysis

Statistical significances based on one-way ANOVA were performed with Prism 5.01 software (GraphPad Software).

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### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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## Conflict of interest

The authors declare that they have no conflict of interest.

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