The IMMUNE-ASSOCIATED NUCLEOTIDE-BINDING 9 Protein Is a Regulator of Basal Immunity in *Arabidopsis thaliana*

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Accepted 27 June 2018.

A robust regulation of plant immune responses requires a multitude of positive and negative regulators that act in concert. The immune-associated nucleotide-binding (IAN) gene family members are associated with immunity in different organisms, although no characterization of their function has been carried out to date in plants. In this work, we analyzed the expression patterns of IAN genes and found that *IAN9* is repressed upon pathogen infection or treatment with immune elicitors. *IAN9* encodes a plasma membrane-localized protein that genetically behaves as a negative regulator of immunity. A novel *ian9* mutant generated by CRISPR/Cas9 shows increased resistance to *Pseudomonas syringae*, while transgenic plants overexpressing *IAN9* show a slight increase in susceptibility. In vivo immunoprecipitation of IAN9-green fluorescent protein followed by mass spectrometry analysis revealed that IAN9 associates with a previously uncharacterized C3HC4-type RING-finger domain-containing protein that we named IAN9-associated protein 1 (IAP1), which also acts as a negative regulator of basal immunity. Interestingly, neither *ian9* or *iap1* mutant plants show any obvious developmental phenotype, suggesting that they display enhanced inducible immunity rather than constitutive immune responses. Because both *IAN9* and *IAP1* have orthologs in important crop species, they could be suitable targets to generate plants more resistant to diseases caused by bacterial pathogens without yield penalty.

The plant immune system comprises an intricate network of receptors and regulators aimed at keeping the cellular homeostasis in the absence of pathogen threat and responding rapidly to biotic stimuli in order to prevent infection. Plants have evolved to perceive pathogen-derived molecules that constitute signals of a potential invasion, also called invasion patterns (Cook et al. 2015). Conserved microbial molecules constitute good targets for recognition by plants; some of these molecules have been shown to be perceived by plant cells as pathogen-associated molecular patterns (PAMP) (Boller and Felix 2009). PAMP are perceived at the cell surface by transmembrane pattern-recognition receptors (PRR) (Zipfel 2014). PRR act in coordination with several regulators and additional proteins that mediate signal transduction (Couto and Zipfel 2016), including mitogen-activated protein kinases (MAPK), calcium-dependent protein kinases, receptor-like cytoplasmic kinases, and respiratory burst oxidase homologs (Bigeard et al. 2015; Macho and Zipfel 2014). Downstream responses include the production of the phytohormone salicylic acid (SA) and antimicrobial compounds, the deposition of callose at the cell wall, and extensive transcriptional reprogramming (Boller and Felix 2009). The activation of immunity is aimed at preventing the proliferation of the perceived pathogen, and prepares plant cells to mount an efficient defense response against subsequent biotic threats. However, defense is costly, in terms of energy investment and the concomitant disruption to the normal developmental program (Huot et al. 2014; Stael et al. 2015), and, as such, needs to be tightly regulated. For this reason, immune responses are inducible, and negative regulators ensure a firm control of their activated state. On the other hand, it has been demonstrated that activation of defense and inhibition of growth can be uncoupled, so that active defense and growth can occur simultaneously, indicating that developmental alterations are the consequence of an active process and not necessarily of limiting resources (Campos et al. 2016; de Wit et al. 2013; Scheres and van der Putten 2017).

Pathogens have developed strategies to manipulate plant cells in order to proliferate inside plant tissues. These include the suppression of plant immunity, the alteration of the physical environment, and the acquisition of nutrients to support their pathogenic lifestyle (Win et al. 2012). In gram-negative bacterial pathogens, the most important virulence factor is the type-III secretion system, which injects effector proteins directly into plant cells (type-III-secreted effectors [T3E]). The manipulation of plant cellular functions by T3E is essential for bacteria to proliferate and is required for the development of

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Funding: This work was supported by the Shanghai Center for Plant Stress Biology (Chinese Academy of Sciences). Research in the Macho laboratory is also supported by the National Natural Science Foundation of China (NSFC) (grant 31571973) and the Chinese 1000 Talents Program. Research in the Lozano-Durán laboratory is also supported by the NSFC (grant 31671994) and the 100 Talents Program from the Chinese Academy of Sciences. C. Caceres-Moreno is sponsored by a CAS-TWAS President's Fellowship for International Ph.D. Students. T. Rosa-Diaz is the recipient of a President's International Fellowship Initiative (PIFI) postdoctoral fellowship (number 2016PB042) from the Chinese Academy of Sciences.

*The e-Xtra logo stands for “electronic extra” and indicates that 21 supplementary figures and three supplementary tables are published online.

disease (Macho 2016; Macho and Zipfel 2015). However, some plants have evolved intracellular receptors that can perceive T3E activities as an indication of pathogen invasion, hence becoming resistant to bacterial infection. These receptors contain nucleotide-binding and leucine-rich repeat domains (NLR) (Khan et al. 2016). NLR activation contributes to the development of defense responses similar to those established after PRR activation but are often more intense and sometimes lead to local cell death, which prevents further pathogen proliferation and spread (Chiang and Coaker 2015). Both PRR and NLR constitute the basis of plant innate immunity, and are the major determinants of basal immunity in plants.

Guanosine triphosphate (GTP)-binding proteins are regulators of various biological processes in eukaryotic cells such as signal transduction, cell proliferation, cytoskeletal organization, and intracellular membrane trafficking, and are classified into numerous families (Takai et al. 2001; Vernoud et al. 2003). IMMUNE-ASSOCIATED NUCLEOTIDE-BINDING/GTPases OF IMMUNITY-ASSOCIATED PROTEINS (IAN/GIMAP) comprise a subfamily of GTPase-like proteins that has been found in anthozoans, vertebrates, and angiosperm plants (Nitta and Takahama 2007; Weiss et al. 2013). In vertebrates, proteins from the IAN/GIMAP family regulate the development and homeostasis of T cells and are associated with autoimmunity (Nitta and Takahama 2007). The transcriptional regulation of genes encoding IAN/GIMAP has been linked to immunity in different organisms: in mice, *IAN/GIMAP* genes are mostly

expressed in immune tissues (Nitta et al. 2006), and they have also been reported as induced in corals after treatment with the bacterial immune elicitor muramyl dipeptide (Weiss et al. 2013). In *Arabidopsis*, IAN/GIMAP are defined by the presence of an *avrRpt2*-induced gene 1 (AIG1) domain, and contain conserved GTP-binding domains, including a P-loop motif known to bind GTP/GDP in Ras GTPases (Bourne et al. 1991) and coiled-coil motifs (Liu et al. 2008). Originally, *AtAIG1* (also known as *IAN8*) was defined as a gene overexpressed in response to the avirulent bacterial strain *Pseudomonas syringae* pv. *maculicola* expressing the effector AvrRpt2 (Reuber and Ausubel 1996). Additionally, computational analysis of transcriptomic data has revealed that the transcription of other IAN family members responds to different biotic stimuli: nematode infection induces the expression of *IAN3* and *IAN11*, while the transcription of *IAN11*, *IAN12*, and *IAN13* is reduced by infection with *Myzus persicae* (Liu et al. 2008).

Despite the accumulating evidence that associates IAN genes with immunity in plants, no characterization of their function in this process has been carried out to date. Here, we analyzed the expression patterns of IAN genes and found that the expression of *IAN9* is repressed upon pathogen infection or treatment with immune elicitors. Further characterization indicated that *IAN9* encodes a plasma membrane (PM)-localized protein and that it genetically behaves as a negative regulator of immunity. In vivo immunoprecipitation (IP) of IAN9-green fluorescent protein (GFP) followed by mass spectrometry (MS) analysis revealed that IAN9 associates with a C3HC4-type RING-finger domain-containing protein that we named IAN9-associated protein 1 (IAP1). Interestingly, our results show that IAP1, like IAN9, negatively regulates immunity, raising the idea that these two proteins may work together in the control of immune responses.

RESULTS

Expression analysis of the IAN gene family upon bacterial infection reveals differential expression patterns for IAN7, IAN8, and IAN9.

The IAN protein family in *Arabidopsis* is composed of 13 members (Fig. 1A) (Liu et al. 2008). Phylogenetic analysis of IAN amino acid sequences shows a clear separation into different subgroups (Fig. 1A; Supplementary Fig. S1). Previous reports have described changes in expression of IAN gene family members upon different biotic stimuli (Liu et al. 2008; Reuber and Ausubel 1996). In order to characterize the transcriptional response of IAN genes to bacterial infection, we inoculated 9- and 10-day-old *Arabidopsis* seedlings with the virulent pathogen *P. syringae* pv. *tomato* DC3000. Three IAN genes showed differential expression patterns upon bacterial infection: *IAN7* and *IAN8* showed a significant upregulation, while *IAN9* showed a significant downregulation (Fig. 1B). We did not detect mRNA for *IAN1*, -2, -4, -6, -10, -11, -12, or -13, suggesting that these genes are not expressed in 10-day-old *Arabidopsis* seedlings in our experimental conditions. The pathogen-induced upregulation of *IAN8* is reminiscent of the originally reported upregulation of this gene by bacteria expressing the effector AvrRpt2, which induces activation of the plant NLR RPS2 (Reuber and Ausubel 1996). Accordingly, we found that the differential expression of *IAN7* or -8 and *IAN9* also takes place upon infection of *Arabidopsis* rosette leaves with *P. syringae* pv. *tomato* expressing AvrRpt2 (Fig. 1C). The particular expression pattern of *IAN9* among the IAN gene family suggests an exclusive function for IAN9 rather than functional redundancy with other IAN family members. This idea is supported by the fact that *IAN9* constitutes a specific phylogenetic group within the IAN gene family (Fig. 1A). For these reasons, we decided to focus our attention on IAN9.

Fig. 1. Transcription patterns of the immune-associated nucleotide-binding (IAN) gene family in *Arabidopsis*. **A**, Phylogenetic tree of IAN proteins. The scale bar denotes a relative measure of evolutionary distance. **B** and **C**, Relative expression of IAN7, IAN8, and IAN9 in 9- to 10-day-old *Arabidopsis* seedlings, 6 h after inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 (**B**) or DC3000 (AvrRpt2) (**C**). Real-time quantitative polymerase chain reaction results were normalized with UBIQUITIN 10 (UBQ10, AT4G05320). Values are means $\pm$ standard error of the mean ($n = 3$ biological replicates; see Materials and Methods). Statistical differences were calculated using one-way analysis of variance ($P < 0.01$). Each experiment was performed three times with similar results.

IAN9 expression is reduced upon chemical activation of plant immunity.

IAN9 is broadly expressed in cotyledons, hypocotyls, and roots of *Arabidopsis* seedlings (Supplementary Fig. S2). To dissect the bacteria-induced repression of *IAN9* transcription, we sought to determine whether perception of purified immune elicitors affects *IAN9* expression. For this purpose, we first treated *Arabidopsis* seedlings with the flagellin-derived peptide flg22, which is widely used as an elicitor of immune responses. Our results show that *IAN9* expression is significantly reduced 1 h after flg22 treatment (Fig. 2A). The perception of different invasion patterns, including flg22, leads to the production of the phytohormone SA, which is a key player in the activation of plant immunity against biotrophic pathogens (Vlot et al. 2009). Treatment with SA for 3 h led to a reduction of *IAN9* expression, although the abundance of *IAN9* transcripts returned to basal levels after a 6-h SA treatment (Fig. 2B), revealing a transient downregulation of *IAN9* transcript abundance upon SA treatment. Finally, to determine whether the reduction on *IAN9* transcription upon flg22 treatment depends on the downstream SA accumulation (Tsuda et al. 2008), we performed flg22 treatment in the SA-depleted *sid2/NahG* plants, deficient in pathogen-induced SA biosynthesis (Wildermuth et al. 2001) and constitutively expressing the bacterial salicylate hydroxylase *NahG*, which degrades SA (Delaney et al. 1994). Interestingly, the flg22-triggered downregulation of *IAN9* transcript abundance was also observed in *sid2/NahG* plants (Fig. 2C), suggesting that SA is not required for the flg22-induced transcriptional repression of *IAN9*.

IAN9 localizes to the PM through its C terminus.

In order to investigate the subcellular localization of *IAN9*, we generated stable transgenic *Arabidopsis* lines expressing an N-terminal GFP-tagged *IAN9* protein (see below for a detailed characterization of these lines), and used confocal microscopy to determine the localization of GFP-*IAN9*. Contrary to free GFP, which shows a nuclear/cytoplasmic localization, GFP-*IAN9* specifically localized at the cell periphery (Fig. 3A). This localization was similar to that observed for well-characterized PM-localized proteins, such as the brassinosteroid (BR) receptor BRI1 (Wang et al. 2001) (Fig. 3A). To determine whether GFP-*IAN9* is associated with membranes, we used the lipophilic fluorescent dye FM4-64, which is rapidly incorporated into membranes upon contact with plant cells (Bolte et al. 2004; Fischer-Parton et al. 2000). Our results show that GFP-*IAN9* fluorescence colocalizes with FM4-64-labeled

compartments (Fig. 3B and C), similar to BRI1-GFP fluorescence and different from free GFP (Fig. 3B and C). To further confirm that *IAN9* localizes at the PM, we performed plasmolysis assays by treating plant tissues with 1 M NaCl. Upon plasmolysis, both GFP-*IAN9* and BRI1-GFP were detected in Hechtian strands, which represent sites of incomplete PM retraction from the cell wall (Fig. 3D). Altogether, our microscopy analysis indicates that *IAN9* localizes at the PM in plant cells.

The C-terminal domain is not conserved among *IAN* proteins. Interestingly, the C-terminal sequence of *IAN9* shows an overrepresentation of positively charged amino acids (KKLRENLERAEKETKELQKKLGKCNIL; 33.3% of R/K), not present in other *IAN* proteins (Supplementary Fig. S3), which could mediate an interaction with the negatively charged phospholipids of the PM. To test this hypothesis, we generated *Arabidopsis* stable transgenic lines expressing a truncated GFP-*IAN9* version lacking the 27 C-terminal amino acids (*IAN9*ΔC-27). The *IAN9*ΔC-27 version lost the specific PM localization seen for wild-type GFP-*IAN9*, and was mostly detected in the cytoplasm. Additionally, we found that, when GFP is fused to the C-terminal end of *IAN9* (*IAN9*-GFP), this protein loses its specific PM localization, and is mostly found in the cytoplasm. Compared with *IAN9*, the *IAN8* C terminus has a lower representation of positively charged amino acids (18.5%), and we found that an N-terminal GFP-tagged *IAN8* (GFP-*IAN8*) localizes to the cytoplasm. Altogether, these results suggest that the exclusive C-terminal domain of *IAN9* is required for its localization at the PM.

Generation of *IAN9* knock-out and overexpression lines.

Public repositories for *Arabidopsis* T-DNA insertion lines contain four independent lines with T-DNA insertions in the *IAN9* genomic locus: *SAIL_167_B02*, *SALK_534_B01*, *SALK_144369*, and *GK-146B08*. For all of these lines, the insertion site is located in the predicted promoter region of *IAN9*. Among them, *GK-146B08* is the line harboring the nearest T-DNA insertion to the *IAN9* start codon (Supplementary Fig. S4) and, therefore, we chose this line for further analyses. Sequencing analysis results showed that the insertion site is located 76 bp upstream of the 5' untranslated region of the *IAN9* gene, and 319 bp upstream of the *IAN9* start codon. Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (RT-qPCR) analyses showed that this mutant has approximately a threefold reduction in the amount of *IAN9* transcripts, indicating that this line is a knockdown *ian9*

Fig. 2. Treatment with flg22 or salicylic acid causes a reduction on the transcription of immune-associated nucleotide-binding (*IAN9*). Relative expression of *IAN9* in 9- to 10-day-old *Arabidopsis* seedlings. WT = wild type. **A** and **C**, Seedlings were treated with 100 nM flg22 for 1 h. **B**, Seedlings were treated with 0.5 mM salicylic acid for 3 or 6 h. Real-time quantitative polymerase chain reaction results were normalized with UBIQUITIN 10 (UBQ10, AT4G05320). Values are means $\pm$ standard error of the mean ($n = 3$ biological replicates; see Materials and Methods). Statistical differences were calculated using one-way analysis of variance ($P < 0.01$). Each experiment were performed three times with similar results.

mutant. To further characterize this line, we determined the *IAN9* transcript levels upon bacterial inoculation. Surprisingly, we found that *IAN9* transcript levels increased significantly upon inoculation with *P. syringae* pv. *tomato* or AvrRpt2, reaching higher levels than those observed in wild-type plants (Fig. 1). These findings indicate that the T-DNA insertion in the *IAN9* promoter generates an aberrant expression pattern of *IAN9* in this line, rendering it unusable for the functional characterization of this gene.

In order to perform genetic analysis of the contribution of *IAN9* to plant immunity, we generated *ian9* mutant lines using CRISPR/Cas9-assisted genome editing (Feng et al. 2013; Mao et al. 2013). We selected the best predicted target site in the *IAN9* gene sequence for recognition by the Cas9/sgrRNA complex (Supplementary Fig. S5), and performed the targeted mutagenesis as explained below (Materials and Methods). Sequencing of the resulting line showed an addition of 1 bp in the second exon of *IAN9*, creating a premature stop codon 366 bp downstream of the start codon (Supplementary Fig. S6), which generates a truncated protein with a disrupted GTP-binding domain. Upon selection of seedlings containing the *ian9* mutation in homozygosis, we selected a line in which the Cas9 gene was segregated out; this Cas9-free *ian9* line was used for further experiments. Additionally, as mentioned before, we generated *Arabidopsis* transgenic lines overexpressing *IAN9* in a Col-0 wild-type background, using a 35S promoter to express a GFP-*IAN9* fusion. We selected two independent homozygous lines that accumulated detectable amounts of GFP-*IAN9* fusion protein, and higher transcript levels of *IAN9* compared with

those in the Col-0 wild type (overexpression [OE]-*GFP-IAN9*#3-10 and OE-*GFP-IAN9*#7-1). As controls, we selected two independent homozygous lines expressing free GFP, which did not show changes in *IAN9* transcription and accumulated detectable levels of free GFP. Interestingly, although the 35S promoter led to high *IAN9* expression compared with the Col-0 wild type, bacterial infection still caused a reduction in *IAN9* transcript levels (Supplementary Fig. S7), suggesting a post-transcriptional regulation of the abundance of *IAN9* transcripts. Overexpression of *IAN9* did not affect *IAN8* expression in basal conditions or upon bacterial infection.

Plant growth and early immune responses are not affected by altered expression of *IAN9*.

Both *IAN9*-overexpressing or *ian9* knockout seedlings and adult plants were visually indistinguishable from the wild type (Supplementary Figs. S8 and S9). Given the predicted association of the IAN family with immune responses and the alteration in *IAN9* expression upon elicitation with flg22, we sought to determine whether *IAN9* is involved in early PAMP-triggered immunity (PTI) responses; namely, the flg22-triggered burst of reactive oxygen species (ROS) and the activation of a cascade of MAPK (Boller and Felix 2009, Macho and Zipfel 2014). Our results show that mutation or overexpression of *IAN9* did not have a detectable impact in the dynamics or total accumulation of ROS upon flg22 treatment (Supplementary Fig. S10). Similarly, neither mutant nor OE lines showed differences in flg22-triggered MAPK activation compared with wild-type plants or free GFP-expressing

Fig. 3. Green fluorescent protein (GFP) immune-associated nucleotide-binding (IAN)9 localizes to the plasma membrane in *Arabidopsis*. **A**, Confocal images of GFP, GFP-*IAN9*, and BRI1-GFP in *Arabidopsis* cotyledon epidermal cells. Bar = 10 μ m. **B**, Confocal images of GFP, GFP-*IAN9*, and BRI1-GFP in *Arabidopsis* cotyledon epidermal cells. *Arabidopsis* seedlings were treated FM4-64 for 5 min before confocal imaging, and the FM4-64 signal is shown in red. Bar = 5 μ m. **C**, Fluorescence intensity through the thin lines shown in B. **D**, Confocal images of GFP, GFP-*IAN9*, and BRI1-GFP in *Arabidopsis* cotyledon epidermal cells after plasmolysis (5-min treatment with 1 M NaCl). Arrows indicate the presence of Hechtian strands. Bar = 10 μ m.

controls. Taken together, these results suggest that alterations of *IAN9* expression do not affect early PTI responses.

IAN9 negatively regulates plant immunity against *P. syringae* pv. *tomato* DC3000.

To test whether *IAN9* contributes to plant immunity against bacterial pathogens, we performed surface inoculation of the *ian9* mutant line with *P. syringae* pv. *tomato* DC3000 and determined bacterial replication in plant tissues. Our results show that knockout mutation of *IAN9* increased plant resistance against DC3000, causing a 13-fold reduction of bacterial titers (Fig. 4A). This increase in disease resistance does not seem to be caused by differences in basal expression of SA-dependent defense-related genes (Supplementary Fig. S11). In order to test whether the increase in resistance also occurs in a context of effector-triggered immunity, we syringe infiltrated *Arabidopsis* rosette leaves with DC3000 (AvrRpt2). However, no differences were detected in terms of replication of this strain (Fig. 4B). To confirm that the observed increased resistance to DC3000 is really due to the absence of *ian9*, we generated complementation lines expressing *GFP-IAN9*, driven by a 35S promoter, in the *ian9* knockout mutant background, where the GFP-*IAN9* protein accumulated and localized to the PM (Supplementary Fig. S12). Expression of GFP-*IAN9* in the *ian9* background was able to rescue the level of growth of DC3000 to that observed in wild-type plants, confirming the association of *IAN9* with the observed increased resistance. Interestingly, transgenic lines overexpressing GFP-*IAN9* in a wild-type background showed a tendency to support higher bacterial loads compared with wild-type or GFP-expressing lines, suggesting that *IAN9* overexpression suppresses plant immunity against DC3000, although such a tendency was not always reproducible or statistically significant across eight independent biological repeats (Supplementary Fig. S13). This trend was not observed when plants were inoculated with a hypovirulent DC3000 derivative unable to produce the virulence factor coronatine (DC3000 COR-) (Supplementary Fig. S14) (Melotto et al. 2006).

Identification of IAN9-interacting proteins.

To characterize further the mode of action of *IAN9*, we searched for proteins physically associated with *IAN9* in plant cells using *Arabidopsis* seedlings expressing *GFP-IAN9* (line OE-*GFP-IAN9*#3-10) and seedlings expressing free GFP as control. Upon GFP IP using agarose beads coupled to an anti-GFP nanobody (GFP-Trap beads), we detected associated proteins using liquid chromatography coupled to tandem MS (LC-MS/MS). In order to detect potential dynamic interactions, we additionally treated seedlings for 1 h with flg22 or SA before IP. Two independent biological replicates showed a large number of proteins physically associated with *IAN9*, which we filtered using the following criteria: (i) presence in both biological replicates, (ii) detection of two or more exclusive unique peptides, and (iii) absence in the GFP control. After applying these filters, 14 proteins were identified as *IAN9* candidate interactors (Supplementary Table S1).

Among these candidate interactors, we drew our attention to an uncharacterized protein encoded by the AT1G18660 gene, which we named IAP1. Although our experimental approach does not provide a quantitative assessment of protein interactions, we noticed that the number of detected IAP1 peptides decreased in samples treated with SA, whereas it did not change substantially in samples treated with flg22. Domain analysis predicts the presence of three tetratricopeptide-like helical (TPR) domains, a C3HC4-type RING-finger domain, and an ATP-dependent protease La (LON) domain in this protein (Supplementary Fig. S15). Upon *Agrobacterium*-mediated transient expression in *Nicotiana benthamiana*, a GFP-IAP1

fusion protein localized at the cell periphery, nucleus (weakly), and around the nucleus; the latter most likely corresponds to the usual endoplasmic reticulum localization of PM-localized proteins overexpressed in this system. Coexpression with red fluorescent protein (RFP)-*IAN9* showed that both proteins colocalize upon transient expression (Fig. 5A). To confirm the interaction between *IAN9* and IAP1, we performed a targeted co-IP using GFP-IAP1 and an N-terminal fusion of *IAN9* to the C-terminal domain of luciferase (Cluc-*IAN9*). Co-IP assays show that GFP-IAP1 interacts with Cluc-*IAN9* but not with Cluc alone (Fig. 5B); Cluc-*IAN9* does not interact with free GFP (Supplementary Fig. S16). To determine whether the *IAN9*-IAP1 interaction is direct, we performed a luciferase complementation assay, transiently coexpressing Cluc-*IAN9* and IAP1 fused to the N-terminal domain of luciferase (IAP1-Nluc). A positive control coexpressing AtSGT1b-Nluc and Cluc-AtRAR1 showed a strong luciferase signal (Supplementary Fig. S17), as described before (Chen et al. 2008). In contrast, tissues coexpressing Cluc-*IAN9* and Nluc-IAP1 did not show any detectable luciferase signal, although both proteins accumulated. As an alternative technique to detect direct interactions, we employed fluorescence resonance energy transfer–fluorescence-lifetime imaging microscopy (FRET-FLIM) by coexpressing GFP-IAP1 and RFP-*IAN9*. However, no difference in lifetime GFP fluorescence was detected when compared with control samples. Although an influence of the tags cannot be ruled out, these results suggest that the interaction observed for IAP1 and *IAN9* is most likely indirect.

IAP1 negatively regulates plant immunity against *P. syringae* pv. *tomato* DC3000.

Public repositories for *Arabidopsis* T-DNA insertion lines contain two independent lines with T-DNA insertions in the *IAP1* genomic locus: *SALK_119114* and *SALK_093498* (Supplementary Fig. S18). We isolated homozygous lines containing these insertions and confirmed the absence of *IAP1* transcripts, naming these lines *iap1-1* and *iap1-2*, respectively. Both lines displayed wild-type-like growth and development when grown on soil under short-day conditions (Fig. 6A), although mutant seedlings showed a slightly reduced root length compared with the Col-0 wild type when grown in vertical MS plates (Supplementary Fig. S19).

Fig. 4. Immune-associated nucleotide-binding (*IAN9*) negatively regulates plant immunity against *Pseudomonas syringae* pv. *tomato* DC3000. **A**, Growth of surface (spray)-inoculated DC3000 (optical density at 600 nm [OD_{600}] = 0.1) in wild-type (WT) Col-0 and *ian9* mutant plants, 3 days postinoculation (dpi). Experiments were repeated more than three times with similar results. **B**, Growth of DC3000 (AvrRpt2) (OD_{600} = 0.001) infiltrated with a needleless syringe into WT Col-0 and *ian9* mutant plants, 3 dpi. Experiments were performed twice with similar results. Data were represented as means $\pm$ standard error (n = 8 independent plants) (A and B). Statistical differences were calculated using a Student's *t* test; "ns" indicates no significant difference and an asterisk indicates significant difference (P < 0.05).

To determine whether mutations in *IAP1* have an impact on plant resistance against bacterial pathogens, we performed surface inoculation of the *iap1* mutant lines with *P. syringae* pv. *tomato* DC3000. Our results show that both *iap1-1* and *iap1-2* mutant lines are more resistant than the Col-0 wild type against DC3000, showing a 19-fold reduction in bacterial loads (Fig. 6B). However, none of these lines showed differences after inoculation with *P. syringae* pv. *tomato* expressing AvrRpt2 (Fig. 6C). This enhancement of disease resistance did not seem to be caused by differences in basal expression of SA-dependent defense-related genes. Interestingly, these results resemble those obtained during the characterization of the *ian9* knockout mutant line (Fig. 4), suggesting that both proteins are involved in the negative regulation of basal immunity against bacterial pathogens, and may act in the same pathway through physical association.

DISCUSSION

In this work, we characterized the transcription patterns of *IAN* family members in *Arabidopsis* upon infection with the bacterial pathogen *P. syringae* pv. *tomato* DC3000. *IAN7* and *IAN8*, which seem to form a separate phylogenetic subgroup, are upregulated upon bacterial infection. In contrast, *IAN9* seems to form a specific subgroup and shows a unique downregulation upon bacterial infection. Infection with a virulent bacterial pathogen such as DC3000 triggers changes in plant cells caused by bacterial virulence activities (e.g., alteration of plant processes by T3E) and basal immune responses. Therefore, formally, transcriptional changes upon infection could be associated with bacterial virulence or plant immunity. In this case, the downregulation of *IAN9* expression upon bacterial infection seems associated with the activation of immunity, because we detected similar expression patterns upon treatment with elicitors of immunity; namely, flg22 and SA. Interestingly, we detected a reduction in the amount of *IAN9* transcripts after bacterial infection even in transgenic lines where *IAN9* overexpression is driven by a constitutive 35S promoter, suggesting a posttranscriptional regulation of *IAN9* transcript abundance. Although the perception of flg22 leads to the production of SA

(Tsuda et al. 2008), our data show that the flg22-triggered reduction of *IAN9* transcription takes place in *sid2/NahG* plants, indicating that this transcriptional change is independent of the observed SA-triggered reduction of *IAN9* transcription. SA-deficient mutants are partially affected in flg22-triggered induction of defense-related genes, probably caused by lower levels of the flg22 receptor FLS2 (Yi et al. 2014). Our results indicate that these lower levels of FLS2 in *sid2/NahG* plants are nevertheless sufficient to trigger the *IAN9* transcriptional repression, suggesting that *IAN9* transcription may be highly sensitive to external biotic stimuli.

In addition to showing different expression patterns, the subcellular localization of *IAN9* (mostly at the PM) is also different from that of *IAN8* (mostly in the cytoplasm). This differential localization further suggests distinct functions for these two *IAN* family members. The C-terminal domain of *IAN9*, required for PM localization, is not present in other *IAN* proteins. This could indicate that the *IAN9* mechanism for PM attachment is exclusive within the *IAN* family, although other *IAN* proteins could localize at the PM through different means.

Mutation of *IAN9* causes increased resistance to DC3000, and this phenotype is rescued in complementation lines. It is worth considering the possibility that *IAN9* is guarded by NLR, as has been shown for some regulators of immunity (Khan et al. 2016), therefore explaining the increased resistance in the *ian9* knockout mutant. However, the absence of developmental phenotypes or constitutive immune responses makes this possibility unlikely. Additionally, overexpression lines show a tendency for increased susceptibility, although this phenotype was not always significant or reproducible, and this trend was not observed upon inoculation with a hypovirulent DC3000 derivative strain. The lack of robustness in this phenotype could suggest that *IAN9* transcript abundance is not rate limiting; however, it could also be due to the potential posttranscriptional regulation of *IAN9* transcript abundance that we have seen in the *IAN9* overexpression lines, which show a reduction in the amount of *IAN9* transcripts after bacterial infection. The transient nature of *IAN9* transcriptional repression upon SA treatment, returning to basal levels after 6 h, may indicate that the negative regulation exerted by *IAN9* could be required to

Fig. 5. Immune-associated nucleotide-binding (IAN)9-associated protein 1 (*IAP1*) interacts with *IAN9* in *Nicotiana benthamiana* leaves. **A**, Confocal microscopy images showing the colocalization of green fluorescent protein (GFP)-*IAP1* and red fluorescent protein (RFP)-*IAN9* in *N. benthamiana* leaves. Bar = 10 μ m. **B**, Cluc or Cluc-*IAN9* was coexpressed with GFP-*IAP1* in *N. benthamiana* before immunoprecipitation (IP) using GFP-trap beads. Immunoblots were analyzed using anti-luc or anti-GFP antibody. Molecular weight (kDa) marker bands are indicated for reference. Experiments were repeated three times with similar results.

contribute to the repression of immune responses after immunity has been established.

Using IP followed by LC-MS/MS, we found IAP1 as a protein associated with IAN9. IAP1 contains three TPR domains, a C3HC4-type RING-finger domain, and an LON domain. Upon transient expression, IAP1 colocalizes with IAN9 in *N. benthamiana* cells. Genetic analysis indicates that both IAN9 and IAP1 negatively regulate immunity against DC3000. Given their physical association, it is possible that both proteins belong to a protein complex involved in the negative regulation of basal immunity against bacterial pathogens. Although we confirmed the association between IAN9 and IAP1 using targeted IP, we failed to detect a direct physical interaction using luciferase complementation or FRET-FLIM assays. Specific limitations of interaction techniques may be hindering the detection of a direct interaction; however, these data may also indicate that the interaction between IAN9 and IAP1 is indirect, perhaps mediated by other scaffolding components in the same protein complex. The *Arabidopsis* Interactome Database has reported several interactors for IAP1 in Y2H assays (Supplementary Table S2). Interestingly, several of these interacting proteins are transcription factors, and others have predicted nuclear localization or undergo nucleocytoplasmic relocalization associated with their activity. Considering that IAP1 interacts with several proteins with nuclear activities, it is tempting to speculate that the IAN9/IAP1 complex could associate with transcriptional regulators in the absence of biotic stress, acting as negative regulators of immune responses, and the complex may dissociate upon pathogen infection to allow for the activation of executor immune responses that restrict pathogen proliferation. This hypothetical model is in agreement with the observation that IAN9 does not seem to regulate early elicitor-induced responses (namely, flg22-triggered ROS burst and MAPK activation). Given that *ian9* or *iap1* mutants do not show autoimmunity phenotypes, the final activation of immune responses may require additional activation by other immune regulators, of which the activity could be facilitated by the absence of IAN9 or IAP1.

The identification of sustainable sources of resistance against plant pathogens is essential to minimize crop losses due to diseases. A well-established approach consists on the mutation of negative regulators of immunity, or the so-called susceptibility genes, although this often leads to fitness costs or autoimmunity phenotypes, which obstruct their applicability in agriculture. Disease resistance based on loss-of-function mutations in *mildew resistance locus o (mlo)* genes is one of the best-known examples of this approach (Kusch and Panstruga 2017). However, *mlo* mutations sometimes have pleiotropic effects that may affect plant yield and increase susceptibility to other pathogens (Kusch and Panstruga 2017). Altogether, our results reveal IAN9 and IAP1 as suitable targets for biotechnological approaches to generate crops with increased disease resistance to bacterial pathogens, because both IAN9 and IAP1 have orthologs in agriculturally important crop species (Supplementary Figs. S20 and S21). Interestingly, IAN9 and IAP1 behave as negative regulators of basal immunity, because *ian9* and *iap1* knockout plants show increased resistance to bacterial infection but they do not show obvious differences compared with wild-type plants in terms of size or development. It remains to be determined whether the mutation of IAN9 or IAP1 orthologs in other plant species will have an impact on the plant response to other biotic or abiotic stresses. Alternative strategies to engineer resistance to plant diseases consider the pathogen-induced transcriptional and translational control of immune regulators (Gurr and Rushton 2005; Xu et al. 2017). These strategies show that it is possible to generate plants where the expression of defense-related genes is restricted to cells undergoing pathogen attack, thus avoiding side effects on plant fitness. Current regulations

hinder the use of transgenic plants to generate disease-resistant crops. Our IAN9 mutagenesis approach shows that it is feasible to design CRISPR/Cas9-mediated strategies to obtain stable non-transgenic mutant plants with increased resistance to pathogens and no obvious developmental defects, paving the way to a potential application in breeding for disease resistance.

MATERIALS AND METHODS

Plant materials and growth conditions.

Arabidopsis seed were sterilized with bleach solution (20% bleach and 0.1% Triton X-100) for 2 to 3 min, then washed with sterile water four to five times and sown on solid 1/2 MS medium (2.21 g of Murashige & Skoog basal medium with vitamins, 15 g of sucrose, and 7 g of agar per 1 liter). After stratification at 4°C for 3 days, the plates were transferred to a growth chamber (22°C, 16 h of light, and 8 h of darkness) for germination and growth. For experiments involving mature *Arabidopsis* plants, sterile seed were firstly stratified at 4°C for 3 days, then transferred to soil. Plants for bacterial infection assays were cultivated in a short-day chamber (22°C, photoperiod of 8 h of light and 16 h of darkness, and 65% humidity). Plants for *Agrobacterium* transformation were grown in a long-day growth room (22°C, photoperiod of 16 h of light and 8 h of darkness).

Bacterial infections.

P. syringae pv. *tomato* DC3000 and DC3000 (AvrRpt2) were streaked on selective 1/2 salt Luria-Bertani (LB) plates (10 g of

Fig. 6. Immune-associated nucleotide-binding (IAN)9-associated protein 1 (IAP1) negatively regulates plant immunity against *Pseudomonas syringae* pv. *tomato* DC3000. **A**, Photography of 4-week-old Col-0 wild type (WT), *iap1-1*, and *iap1-2* plants, grown in a photoperiod of 8 h of light and 16 h of darkness. Scale bar is 0.5 cm. **B**, Growth of surface (spray)-inoculated DC3000 (optical density at 600 nm [OD₆₀₀] = 0.1) in wild-type (WT) Col-0, *iap1-1*, and *iap1-2* mutant plants, 3 days postinoculation (dpi). Experiments repeated more than three times with similar results. **C**, Growth of DC3000 (AvrRpt2) (OD₆₀₀ = 0.001) infiltrated with a needleless syringe into wild-type (WT) Col-0, *iap1-1*, and *iap1-2* mutant plants, 3 dpi. Experiments performed twice with similar results. Data were represented as means ± standard error (*n* = 8 independent plants) (B and C). Statistical differences were calculated using a Student's *t* test; "ns" indicates no significant difference and an asterisk indicates significant difference (*P* < 0.05).

tryptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar per 1 liter) and cultivated at 22°C for 2 days. For DC3000, bacteria were collected from the plates into sterile water, the optical density (OD) was adjusted to a value of 0.1 (5×10^7 CFU/ml) or 0.2 (10^8 CFU/ml), and silwet-L77 was added to a final concentration of 0.03% before performing spray inoculation onto 3- to 4-week-old soil-grown *Arabidopsis* under short-day conditions. The plants were then covered with cling wrap for 3 h. The whole plants were harvested in 1.5-ml microcentrifuge tubes and weighed. For DC3000 (AvrRpt2), the OD was adjusted to 0.1 (5×10^7 CFU/ml) or 0.001 (10^5 CFU/ml). The bacterial suspension was pressure infiltrated into 5- to 6-week-old short-day-grown *Arabidopsis* leaves with a needleless syringe (3 leaves/plant), and leaf discs were collected into 1.5-ml tubes using a leaf punch at 3 days postinoculation. In both cases, plant tissues were ground and homogenized in sterile water before plating serial dilutions on selective 1/2 salt LB agar plates. The plates were placed at 28°C for 1.5 days and the bacterial growth was calculated as CFU.

For gene expression assays, sterile seed were sown on 1/2 solid MS medium to germinate and grown for 3 to 4 days in long-day conditions. Seedlings were then transferred into 1/2 liquid MS medium in 12-well culture plates (Thermo Fisher Scientific, Waltham, MA, U.S.A.), and grown for another 6 or 7 days. Each well contained three seedlings (pulled together as one sample), and every experiment included three independent samples from three independent wells. Seedlings were inoculated with DC3000 or DC3000 (AvrRpt2) at 5×10^7 CFU/ml, and samples were collected 6 h after inoculation.

Treatments with immune elicitors.

Sterile seed were sown on 1/2 solid MS medium to germinate and grown for 3 to 4 days in long-day conditions. Seedlings were then transferred into 1/2 liquid MS medium in 12-well culture plates (Thermo Fisher Scientific), and grown for another 6 or 7 days. Each well contained three seedlings (pulled together as one sample), and every experiment included three independent samples from three independent wells. The flg22 peptide or SA were added into the liquid medium to a final concentration of 100 nM and 0.5 mM, respectively. All of the plates were incubated on a shaker for 5 to 10 min following addition of the elicitor. Samples were harvested into 1.5-ml tubes at different time points after treatment, as indicated in the figures.

Root growth assay.

Sterile seed were sown on 1/2 solid MS medium and stratified at 4°C for 3 days. Then, the plates were placed into a growth chamber for 1 day. The germinated seedlings were transferred to the new 1/2 solid MS medium (square plates). Square plates were placed vertically into a growth chamber and root length was measured 5 days later.

RNA isolation, RT-PCR, and RT-qPCR.

For RNA extraction, plant tissues were collected in 1.5-ml microfuge tubes with one metal bead and the tubes were immediately placed into liquid nitrogen. Samples were ground thoroughly with a TissueLyser (QIAGEN, Duesseldorf, Nordrhein-Westfalen, Germany) for 1 min and placed back in liquid nitrogen. Total RNA was extracted with the E.Z.N.A. Plant RNA kit (Omega Bio-Tek, Norcross, GA, U.S.A.) with in-column DNA digestion and an additional sample treatment with DNAase (Omega Bio-Tek). First-strand cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, U.S.A.) with a volume of 20 μ l. For RT-PCR, the PCR was performed in 50 μ l using the Q5 Hot Start High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, U.S.A.) with

35 or 38 cycles. For RT-qPCR, PCR were performed in 20 μ l using the iTaq Universal SYBR Green Supermix kit (Bio-Rad) in the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.). Data were analyzed with Excel and GraphPad Prism 6.

Confocal microscopy imaging.

Cotyledons from 3- to 4-day-old long-day-grown *Arabidopsis* seedlings were imaged using a Confocal Laser Scanning Microscope Platform: Leica TCS SP8 (Leica, Mannheim, Germany). The GFP was excited with an argon laser at 488 nm, and its emission was detected at 500 to 550 nm. For plasmolysis assays, cotyledons were placed into a 1 M NaCl solution on glass slides, and GFP was observed and recorded after 5 to 10 min. For FM4-64 staining, cotyledons from 4-day-old seedlings were cut and soaked into FM4-64 solution at 5 ng/ μ l as described previously (Beck et al. 2012) for 5 min; samples were then transferred to water on glass slides and covered with coverslips. For dual-channel simultaneous observation, the fluorescence signal of FM4-64 was excited with an argon laser at 561 nm and its emission was observed at 580 to 650 nm; the GFP was excited at 488 nm and observed at 500 to 550 nm. For colocalization assays, 3-week-old *N. benthamiana* leaves were coinfiltrated with *Agrobacterium* GV3101 (pMP90) carrying plasmids to express *RFP-IAN9* and *GFP-IAP1*. Leaves were coinfiltrated with the GV3101 strain carrying plasmids to express *RFP-IAN9* and *GFP* as control. Leaf discs (3 mm in diameter) were punched from the whole leaf 24 h post-infiltration and transferred to water on glass slides. For dual-channel image acquisition, GFP and RFP were excited at 488 and 561 nm respectively; emission was collected at 500 to 550 nm for GFP and 580 to 650 nm for RFP.

MAPK activation and Western blot assays.

For protein extraction, *Arabidopsis* seedlings or leaf discs from *N. benthamiana* were ground with a Tissue Lyser (QIAGEN, Hilden, Nordrhein-Westfalen, Germany). Samples were then resuspended in lysis buffer (2 $\times$ loading buffer: 100 mM Tris-HCl [pH 6.8], 10% Glycerol, 2% sodium dodecyl sulfate [SDS], and 0.03% bromophenol blue), boiled at 95°C for 10 min, and centrifuged at 14,000 $\times$ g for 5 min before loading in SDS polyacrylamide gel electrophoresis (PAGE) acrylamide gels. Western blots were performed using anti-GFP (Sigma G6795), antiluciferase (Sigma L0159), anti-mouse immunoglobulin G (IgG)-peroxidase (Sigma A2554), and anti-rabbit IgG-peroxidase (Sigma A0545).

MAPK activation assays were performed as previously described (Macho et al. 2012), with minor modifications. Briefly, 7-day-old *Arabidopsis* seedlings grown on solid 1/2 MS medium were transferred to water and then treated with 100 nM flg22 for 10 min after vacuuming for 5 min (15 min in total). AntipMAPK (Phospho-p44/42 MAPK [Erk1/2] [Thr202/Tyr204] XP Rabbit mAb; Cell Signaling 4370) was dissolved in 3% gelatin (Sigma G7041) and used to hybridize the membranes. All membranes were stained with Ponceau stain (Sangon Biotech, Shanghai, China) to verify equal loading.

ROS burst.

ROS burst assays were conducted as previously described (Sang and Macho 2017). Briefly, 4-mm leaf discs from 5- to 6-week-old *Arabidopsis* plants grown in short-day conditions were transferred to 96-well microplates (PerkinElmer, Waltham, MA, U.S.A.) with 100 μ l of Mili-Q water per well and incubated overnight. Water was then removed and ROS burst was elicited by adding 100 μ l of a solution containing 100 ng flg22, 100 μ M luminol, and 20 μ g/ml horseradish peroxidase.

The luminescence was recorded over 40 min using a Thermo Scientific VARIOSKAN FLASH (Thermo Fisher Scientific).

Co-IP and large-scale IP for LC-MS/MS.

Leaves from 3- to 4-week-old *N. benthamiana* plants were coinfiltrated with *Agrobacterium* GV3101 (pMP90) carrying plasmids to express *GFP-IAP1* and *Cluc-IAN9*; leaves coinfiltrated with GV3101 carrying plasmids to express *GFP-AIP1* and *Cluc* or *GFP* and *Cluc* were used as controls. Total proteins were extracted 24 h later and IP was performed with GFP-trap beads (Chromotek, Am Klopferspitz, Planegg-Martinsried, Germany), as described previously (Sang et al. 2018). Proteins were stripped from the beads by boiling in 50 µl of SDS loading buffer for 10 min. Immunoprecipitated proteins were separated on SDS-PAGE acrylamide gels and Western blots were performed as described above. Large-scale IP assays for LC-MS/MS were performed as described before (Kadota et al. 2016; Sang et al. 2018), using 5 g of 10-day-old *Arabidopsis* seedlings before or after treatment with 100 nM flg22 or 0.5 mM SA.

Plasmid construction.

The sequence of all of the primes used in this study is shown in Supplementary Table S3. A free GFP fragment (with stop codon) was amplified from the plasmid pGWB505 (Nakagawa et al. 2007). The purified PCR product was transferred into entry vector pDONR/ZEO (Thermo Fisher Scientific) by BP reaction, then recombined into pGWB602 (35S promoter, no tag) (Nakagawa et al. 2007) using LR reaction to yield the pGWB602-*GFP* plasmid. The coding DNA sequence of *IAN9*, *IAN9* (Δ C27aa), *IAN8*, and *IAP1* (all with stop codon) were amplified from cDNA of whole *Arabidopsis* seedlings using primers containing attB1attB2 sites. The PCR fragments were ligated into pDONR/ZEO by BP reaction, then recombined with the binary vector pGWB606 (35S pro, N-sGFP) (Nakagawa et al. 2007) to yield the plasmids pGWB606-*IAN9*, pGWB606-*IAN9* (Δ C27aa), pGWB606-*IAN8*, and pGWB606-*IAP1* through an LR reaction. pDONR/ZEO-*IAN9* was also recombined with the binary vector pGWB555 (35S promoter, N-mRFP) to yield the plasmid pGWB555-*IAN9* through an LR reaction.

For luciferase complementation assays, the original vectors (Chen et al. 2008) were modified to make them Gateway compatible. The binary vector pCambia1300-nLUC was digested with *SacI* and *SalI* and pCambia1300-cLUC was digested with *KpnI* and *SalI*. The gateway cassette was then amplified from pGWB505 with primers *Nluc-F/R* and *Cluc-F/R* and PCR products were cloned into the digested pCambia1300 vectors using ClonExpress II One Step Cloning Kit (Vazyme Biotech, Nanjing, China) to yield the new version of the binary plasmids pGWB-*Nluc* and pGWB-*Cluc* containing the Gateway cassette. Then, pDONR/ZEO-*IAP1* (without stop codon) and pDONR/ZEO-*IAN9* were separately recombined with pGWB-*Nluc* and pGWB-*Cluc* through an LR reaction to yield the destination vectors pGWB-C3HC4-*Nluc* and pGWB-*Cluc-IAN9*.

For CRISPR/Cas9-mediated mutagenesis, the detailed sites were predicted using the CRISPR Design tool. PCR products were ligated into the pCAS9 plasmid (Feng et al. 2013) using T4 DNA ligase (New England Biolabs).

Arabidopsis transformation.

For the generation of *Arabidopsis* transgenic lines, *Agrobacterium*-mediated transformation was performed according to the method described before (Clough and Bent 1998). The *Agrobacterium tumefaciens* strain GV3101 (pMP90) carrying the desired plasmids (pGWB602-*GFP*, pGWB605-*IAN9*, pGWB606-*IAN9*, pGWB605-*IAN8*, and pGWB606-*IAN9* [Δ C-27aa]) were cultured overnight at 28°C, then spun down and resuspended to an OD at 600 nm (OD₆₀₀) = 1 in 50 ml of

5% sucrose and 0.03% silwet-L77 solution. The fertilized siliques from 5- to 6-week-old Col-0 wild-type plants (grown in a photoperiod of 16 h of light and 8 h of darkness) were removed before flower dipping. After dipping for 1 to 2 min, the plants were covered with polyethylene cling wrap for 16 to 24 h in the dark, then put back to normal growth conditions until seed collection. Finally, homozygous transgenic lines were obtained after resistance selection (BASTA at 15 µg/ml and hygromycin B at 25 µg/ml) and segregation ratio calculation.

For the generation of the *ian9* mutant, *A. tumefaciens* GV3101 carrying the plasmid pCas9 (35S, AtU6, Hpt)-*IAN9* was used to transform Col-0 wild-type *Arabidopsis* plants. T1 plants were selected in 1/2 solid MS (hygromycin B at 25 µg/ml), then transferred to soil. Genomic DNA was extracted from 2-week-old soil-grown T1 plants using the cetyltrimethylammonium bromide method (Doyle and Doyle 1987). *IAN9* was amplified from independent DNA samples and sequenced. The plants that possessed a double peak in the sequencing results of the target sequence were chosen for collection of T1 seed. Independent T1 seed were sown on selective (hygromycin B at 25 µg/ml) and nonselective 1/2 solid MS medium and the segregation ratio was calculated. Lines with 3:1 ratio were transferred to the soil. *IAN9* and *CAS9* genes were amplified by PCR, and plants without the *CAS9* product were selected. Homozygous CRISPR/CAS9 mutant *ian9* lines without *CAS9* background were selected for further experiments.

Agrobacterium-mediated transient expression in *N. benthamiana*.

A. tumefaciens GV3101 carrying the desired plasmid (pGWB606-*IAP1*, pGWB555-*IAN9*, pGWB-*Cluc*, pGWB-*Cluc-IAN9*, or pGWB-*Nluc-IAP1*) were grown on selective LB plates (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 15 g of agar per liter) and cultivated at 28°C for 2 days. Before infiltrating 3- to 4-week-old *N. benthamiana* plants, *Agrobacterium* cells were resuspended in the infiltration buffer (10 mM MgCl₂, 10 mM morpholineethanesulfonic acid [pH 5.6], and 150 µM acetosyringone) directly from plates, and diluted to an OD₆₀₀ of 0.5 or 1, depending on the expression and stability of the different proteins. Samples were collected 24 h after *Agrobacterium* infiltration.

Luciferase complementation assay.

Luciferase complementation assays were performed as previously described (Chen et al. 2008). Three-week-old *N. benthamiana* leaves were coinfiltrated with *Agrobacterium* GV3101 (pMP90) carrying the plasmids to induce the expression of *IAP1-Nluc* and *Cluc-IAN9*. One day after inoculation, the whole leaves were cut and sprayed with luciferin solution (1 mM luciferin [Sigma] and 0.02% Triton X-100). The fluorescence signal was recorded using a Lumazine 1300B (Scientific Instrument, West Palm Beach, FL, U.S.A.) for 10 min after 5 min in the dark.

FRET-FLIM.

Three-week-old *N. benthamiana* leaves were coinfiltrated with *Agrobacterium* GV3101 (pMP90) carrying the plasmids to induce the expression of *GFP-IAP1* with *RFP-IAN9* (FRET pair: donor + acceptor); leaves infiltrated with GV3101 inducing the expression of *GFP-IAP1* with *RFP* (donor + free RFP) and *GFP-IAP1* (donor alone) were used as negative control. FRET-FLIM experiments were performed on a Leica TCS SMD FLCS confocal microscope. Leaf discs (6 mm in diameter) of *N. benthamiana* plants transiently coexpressing donor and acceptor were visualized 1 day after agroinfiltration. The lifetime of the donor (τ) was collected and analyzed as described by Rosas-Diaz et al. (2018).

Chemicals.

The flg22 peptide (TRLSSGLKINSKDDAAGLQIA) was purchased from Abclonal, USA. Sequencing-grade modified trypsin was purchased from Promega Corp. (Madison, WI, U.S.A.). All other chemicals were purchased from Sigma-Aldrich (St. Louis), unless otherwise stated.

ACKNOWLEDGMENTS

We thank members of the Macho and Lozano-Durán laboratories for helpful discussions; J. Li, J.-M. Zhou, and J.-K. Zhu for sharing biological materials; X. Jian for technical and administrative assistance during this work; the PSC Cell Biology core facility for assistance with confocal microscopy; and the PSC Proteomics core facility for LC-MS/MS analysis.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- The Arabidopsis Interactome Database:
http://interactome.dfci.harvard.edu/A_thaliana/index.php
 CRISPR Design tool: <http://crispr.mit.edu/>