

# The developmental selector *AS1* is an evolutionarily conserved regulator of the plant immune response

Pedro L. Nurnberg\*, Kirsten A. Knox\*, Byung-Wook Yun\*, Peter C. Morris†, Reza Shafiei\*, Andrew Hudson\*, and Gary J. Loake\*\*

\*Institute of Molecular Plant Sciences, School of Biological Sciences, University of Edinburgh, King's Buildings, Edinburgh EH9 3JR, United Kingdom; and †School of Life Sciences, Heriot-Watt University, Riccarton, Edinburgh EH14 4AS, United Kingdom

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The MYB-related gene *ASYMMETRIC LEAVES 1* (*AS1*) and its orthologs have an evolutionarily conserved role in specification of leaf cell identity. *AS1* is expressed in leaf founder cells, where it functions as a heterodimer with the structurally unrelated *AS2* proteins to repress activity of *KNOTTED 1-like homeobox* (*KNOX*) genes. *AS1* therefore confines *KNOX* activity to the shoot apical meristem, where it promotes stem cell function through the regulation of phytohormone activities. Here, we show that loss-of-function mutations in *AS1* unexpectedly convey heightened protection against necrotrophic fungi. *AS1* operates as a negative regulator of inducible resistance against these pathogens by selectively binding to the promoters of genes controlled by the immune activator, jasmonic acid (JA), damping the defense response. In contrast, *AS1* is a positive regulator of salicylic acid (SA)-independent extracellular defenses against bacterial pathogens. Neither the absence of *AS2* nor *ERECTA* function, which enhances the morphological phenotype of *as1*, nor the conditional or constitutive expression of *KNOX* genes impacted disease resistance. Thus, the function of *AS1* in responses to phytopathogens is independent of its *AS2*-associated role in development. Loss of function in the *AS1* orthologs *PHAN* in *Antirrhinum majus* and *NSPHAN* in *Nicotiana sylvestris* produced pathogen-response phenotypes similar to *as1* plants, and therefore the defense function of *AS1* is evolutionarily conserved in plant species with a divergence time of  $\approx 125$  million years.

*Arabidopsis thaliana* | asymmetric leaves 1 | disease resistance | plant defense response

An evolutionarily conserved genetic pathway controls specification of leaf cell fate at the shoot apical meristem and involves the *Arabidopsis* *ASYMMETRIC LEAVES1* (*AS1*), *Antirrhinum* *PHANTASTICA* (*PHAN*), *Nicotiana* *NSPHAN*, and maize *ROUGH SHEATH2* (*RS2*) genes. These genes encode orthologous MYB transcription factors that are expressed only in leaf founder cells and leaf primordia, where they are needed to repress *KNOTTED1-like homeobox* (*KNOX*) genes (1–4). *KNOX* genes are expressed within the shoot apical meristem, where they regulate the activity of gibberellin and cytokinin hormones to maintain indeterminacy (5–9). *AS1* forms heterodimers with the structurally unrelated protein *AS2* (10), and both proteins are required to prevent *KNOX* expression in developing leaves (9, 10). Ectopic *KNOX* expression produces lobed leaves, similar to those of *as1* and *as2* loss-of-function mutants (11), suggesting that it is responsible for this aspect of the *as1* or *as2* mutant phenotype.

A small number of genes that are needed for more general aspects of plant development have also been found to regulate disease resistance. For example, a potential transcriptional cofactor encoded by *AtTIP49a* promotes the plant defense response but is also needed for viability of female gametophytes and seedlings (12). Similarly, a potential component of a ubiquitin protein ligase complex encoded by *SGT1b* (*SUPPRESSOR OF THE G2 ALLELE OF skp1-4 1b*) is required for both plant disease resistance and auxin signaling (13).

The emerging evidence suggests that plant immune function discriminates between microbes that exhibit distinct pathogenic lifestyles (14). Thus, salicylic acid (SA)-dependent defenses largely underpin resistance against biotrophic pathogens, whereas jasmonic acid (JA)- and ethylene (ET)-based responses are required for protection against necrotrophic pathogens (15–18). Furthermore, these signaling networks are thought to be regulated in a mutually antagonistic fashion, suggesting that plants can prioritize specific defense mechanisms (19–20). Recently, a series of loss-of-function mutations have been uncovered that convey enhanced susceptibility to necrotrophic fungi (21–23). However, only *ocp3* (*overexpressor of cationic peroxidase 3*) and *jin1* (*jasmonate-insensitive 1*) have been reported to increase resistance against this class of pathogen (24, 25).

Here, we show that loss of *AS1* function conveys increased protection against necrotrophic fungal pathogens. Our findings suggest that *AS1* operates as a negative regulator of inducible defense responses against these pathogens by occupying the promoters of a subset of defense genes, damping their expression. In contrast, *AS1* operates as a positive regulator of disease resistance against bacterial pathogens. These *AS1* functions are independent of its *AS2*- and *KNOX*-associated roles in development and are conserved in plant species with a divergence time of  $\approx 125$  million years.

## Results

**Forward Screen to Uncover Mutations that Convey Resistance Against *Botrytis cinerea*.** To identify *Arabidopsis* mutants expressing increased resistance against the necrotrophic fungal pathogen *B. cinerea*, we screened the progeny of  $\approx 10,000$  plants carrying T-DNA insertions (26) by spraying with a suspension of *B. cinerea* spores. One mutant was found with increased resistance to *B. cinerea* and lobed leaves (Fig. 1 *A–D*). Increased pathogen resistance and altered leaf morphology behaved as a single recessive trait and cosegregated with insensitivity to glufosinate herbicide encoded by the T-DNA. Sequences flanking this insert were recovered by thermal asymmetric interlaced (TAIL)-PCR, revealing that the insertion was within the only intron of the *AS1* gene, and that the T-DNA prevented accumulation of *AS1* transcripts detectable by RT-PCR, consistent with a null mutation (data not shown). The mutation was confirmed as an allele of *AS1* in crosses to the classical mutation *as1-1* (27) and was therefore named *as1-4*. Furthermore, *as1-1* also conferred heightened resistance against *B. cinerea*, confirming the pleio-

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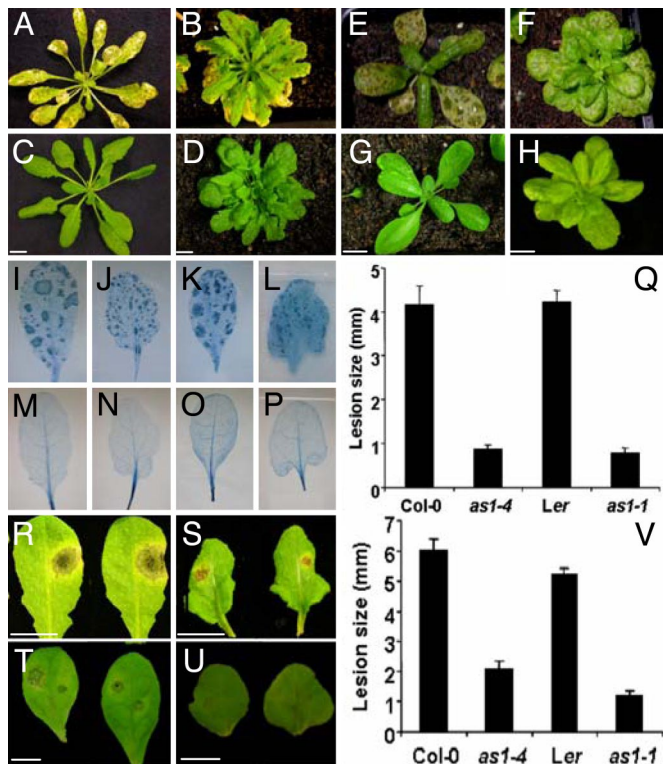
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\*To whom correspondence should be addressed. E-mail: gloake@ed.ac.uk.

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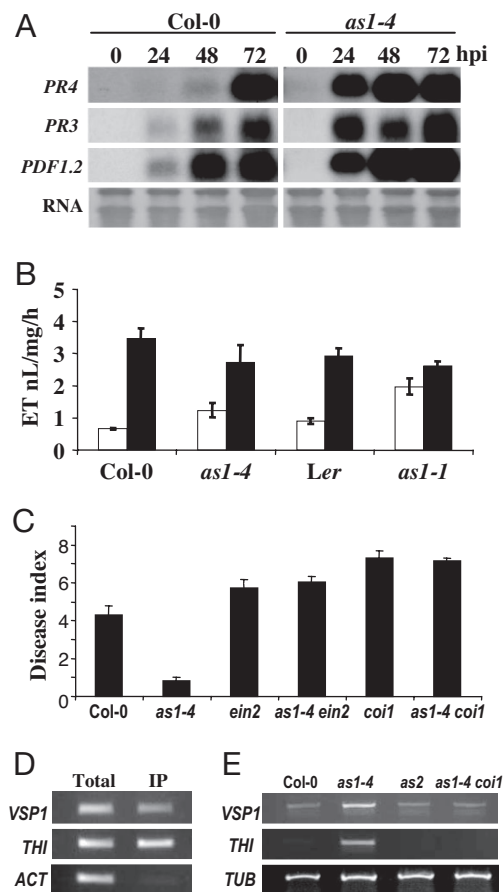


**Fig. 1.** Loss-of-function mutations in *AS1* increase disease resistance against necrotrophic fungi. (A–H) The *Arabidopsis* lines Col-0 (A and C), Col-0 *as1-4* (B and D), *Ler* (E and G), and *Ler as1-1* (F and H) were challenged with a spore suspension of *B. cinerea* (A, B, E, and F). For mock controls, the inoculation solution lacked spores (C, D, G, and H). All plants were scored for disease development at 96 hpi. (I–P) Col-0 (I and M), *as1-4* (J and N), *Ler* (K and O), and *as1-1* plants (L and P) were either challenged with a spore suspension of *B. cinerea* (I–L) or mock inoculated (M–P) as described above. Subsequently, these lines were stained with trypan blue, which marks dead plant cells, revealing lesion development. (Q) Quantification of the extent of *B. cinerea*-generated lesions in the indicated plant genotypes. (R–U) The *Arabidopsis* lines Col-0 (R), Col-0 *as1-4* (S), *Ler* (T), and *Ler as1-1* (U) were drop-inoculated with an *A. brassicicola* spore suspension and scored at 96 hpi. (V) Size of *A. brassicicola*-generated lesions in the indicated plant genotypes. Error bars represent 95% confidence limits. All experiments were repeated at least twice with similar results. (Scale bars, 1 cm.)

tropic roles of *AS1* in pathogen resistance and plant development (Fig. 1 E–H).

We monitored expression of the *B. cinerea ActA* gene, which correlates directly with the extent of *B. cinerea* growth (28). At 96 h postinoculation (hpi), *ActA* transcript accumulation in *as1-1* plants was reduced by 71% compared with wild type (data not shown). Furthermore, trypan blue staining of dead cells after *B. cinerea* inoculation revealed that *as1* mutants developed strikingly smaller lesions (reduced in size by  $\approx 80\%$  compared with wild type; Fig. 1 I–Q). Scoring overall symptom development with a disease index also showed *as1* mutations conveyed robust resistance against *B. cinerea* (data not shown). The level of protection against *B. cinerea* infection in *as1* plants was similar to that established by exogenous application of JA to wild-type plant lines [supporting information (SI) Fig. 5]. To investigate whether *as1* can restrict the growth of other necrotrophic fungi, *as1* and wild-type plants were challenged with *Alternaria brassicicola*. Both *as1-1* and *as1-4* mutants showed increased resistance, marked by a conspicuous decrease in lesion size (Fig. 1 R–V).

***as1*-Mediated Resistance Requires CO11 and EIN2.** Resistance to necrotrophic pathogens is typically dependent on the plant



**Fig. 2.** Misexpression of JA/ET-dependent defense response genes in *as1* plants. (A) Northern blot analysis of *PDF1.2*, *PR3*, and *PR4* transcript accumulation in the indicated plant genotypes challenged with *B. cinerea* at the stated hpi. (B) Determination of the rate of ET production in the indicated plant lines. Noninoculated plants (empty bars) and *B. cinerea*-inoculated plants (filled bars) and scored for disease development at 96 hpi. (C) The stated plant genotypes were challenged with *B. cinerea* and scored for disease development at 96 hpi. (D) Detection of the indicated gene-regulatory sequences by PCR in total and immunoprecipitated (IP) chromatin extracts by using an anti-GFP antiserum to enrich for chromatin containing PHAN-GFP. The actin (*ACT*) gene is used as a nonbinding control. (E) Accumulation of the listed gene transcripts in Col-0 wild-type and *as1* and *as2* mutant plants determined by RT-PCR. Tubulin (*TUB*) is a constitutively expressed control. Error bars represent 95% confidence limits. These experiments were repeated at least twice with similar results.

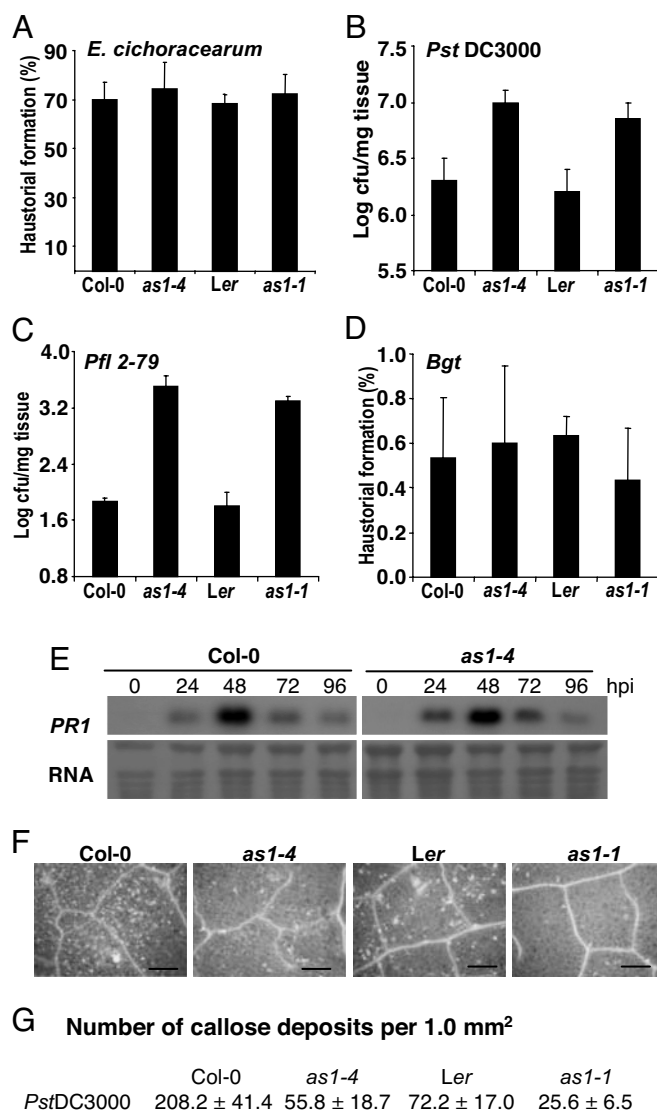
hormones JA and ET (15–18). To examine whether increased resistance in *as1* mutants involved altered JA or ET responses, we monitored expression of defense marker genes that are up-regulated by these hormones. In *as1-4* mutants, induction of the JA/ET-dependent *PLANT DEFENSIN1.2* (*PDF1.2*), *PATHOGENESIS-RELATED3* (*PR3*), and *PR4* genes (15) was accelerated in response to *B. cinerea* infection (Fig. 2A). We therefore determined the concentrations of JA and ET during infection with *B. cinerea*. In noninoculated leaves, levels of ET were twice as high in *as1-1* mutants compared with wild type and 1.3 times higher in *as1-4* mutants but induced to the same levels in *as1* and wild type upon infection with *B. cinerea* (Fig. 2B). JA levels in uninfected and infected *as1* mutants were similar to wild type (data not shown). To examine if ET or JA signaling is required for *as1*-mediated disease resistance, we generated *as1* double mutants with either *ein2* (29) or *coi1* (30), which cause insensitivity toward ET or JA, respectively, and result in enhanced susceptibility to some necrotrophic pathogens (15, 16). In response to *B. cinerea*, *as1 ein2* and, particularly, *as1 coi1* plants

exhibited increased lesion development relative to the *as1* line (Fig. 2C) and were compromised in JA/ET-dependent gene expression (data not shown). Therefore, *as1*-mediated resistance against *B. cinerea* requires ET and, more significantly, JA signaling.

**Identification of Gene Promoters Occupied by AS1.** Because *as1* mutants showed increased induction of pathogen-induced marker genes, we tested whether AS1 might have a direct role in regulation of defense genes by using a chromatin immunoprecipitation (ChIP)-gene chip approach (31). A transgene expressing a PHAN-green fluorescent protein (GFP) fusion complemented both the developmental and defense-related phenotypes of *as1* mutants, revealing that PHAN is functionally equivalent to AS1 (SI Fig. 6). ChIP-chip utilizing antibodies against GFP suggested that AS1 occupied the promoters of at least 96 genes (SI Table 1). We analyzed the expression profiles of these genes, where possible, by interrogating array experiments documented in the TAIR database (www.arabidopsis.org). The expression of 37% of interrogated genes was changed by at least 2-fold in response to *B. cinerea*/JA/ET (SI Table 2), consistent with AS1 having a direct role in regulation of genes that are involved in defense.

The well characterized JA-regulated genes *THIONIN* (*THI*) and *VEGETATIVE STORAGE PROTEIN* (*VSP1*) (17) were among the most significantly enriched target genes in ChIP-chip. Maximum *VSP1* and *THI* enrichment occurred with DNA sequences  $-350$  to  $400$  and  $-150$  to  $250$  bp, respectively, upstream of their respective start codons. In the case of *VSP1*, this sequence is 89 bp upstream of a known JA-responsive element (32). Primers designed to amplify these sequences were used to verify PHAN binding in three independent ChIP experiments, in which *THI* and *VSP1* were enriched 5.39- and 5.42-fold, respectively (Fig. 2D), and similar enrichment was obtained when a different antibody against GFP was used in immunoprecipitation (SI Fig. 7). *THI* and *VSP1* transcript accumulation was greater in *as1* compared with wild type, suggesting that AS1 binding is needed to repress these two target genes (Fig. 2E). *THI* and *VSP1* were not misexpressed in an *as1 coi1* double mutant, indicating that JA signaling is still required to induce *THI* and *VSP* expression in the absence of AS1 activity. In addition, *as2* mutants did not show increased *THI* and *VSP1* transcript levels, suggesting that the role of AS1 in disease resistance might be independent of AS2.

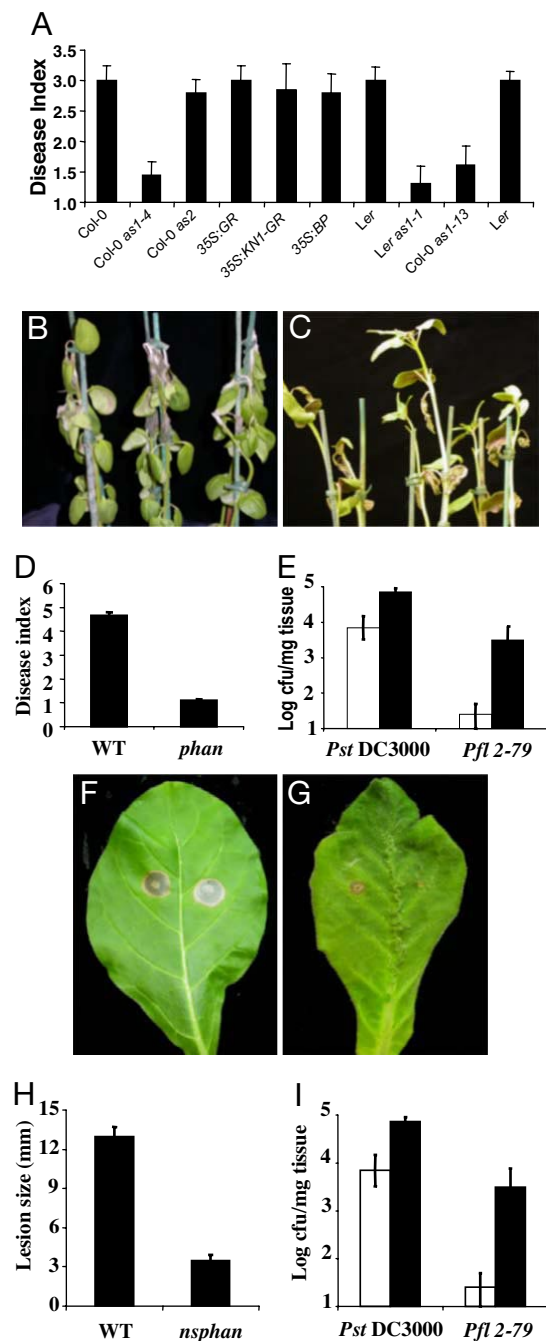
**Response of *as1* Plants to Diverse Pathogens.** We tested whether AS1 function affected responses to other pathogens. Resistance to a virulent biotrophic powdery mildew fungus, *Erysiphe cichoracearum* (33), was unaffected by the *as1* mutation (Fig. 3A). In contrast, *as1* mutants supported growth of the virulent hemibiotrophic bacterium, *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 (34), to levels  $\approx 7$  times greater than wild type (Fig. 3B). Similarly, *as1* mutants were more susceptible to *Pseudomonas fluorescens* (*Pfl*) 2-79, for which wild-type *Arabidopsis* is a nonhost (35) (Fig. 3C). *Arabidopsis* is also a nonhost for the wheat powdery mildew fungus *Blumeria graminis* f.sp. *tritici* (*Bgt*) (36). In contrast to *Pfl*-2-79, however, nonhost resistance against *Bgt* was not compromised in *as1* mutants (Fig. 3D). Basal and resistance (*R*) gene-mediated protection against two isolates of *Hyaloperonospora parasitica*, Noco2 (37) and Cala2 (38), also appeared not to be impacted by mutations in *AS1* (SI Fig. 8). Neither *PR1* expression (Fig. 3E) nor SA accumulation (data not shown) was affected in *as1* plants inoculated with *Pst*DC3000. However, callose deposition, a marker for SA-independent extracellular defense (39), was compromised in response to either *Pst*DC3000 (Fig. 3F and G) or *Pfl*-2-79 (data not shown).



**Fig. 3.** Both nonhost and basal resistance is compromised in *as1* plants against attempted bacterial infection. (A) Extent of *E. cichoracearum* infection measured by determining the percentage of conidia that form a haustorium on the indicated plant lines. (B) Quantification of *Pst*DC3000 growth in the stated plant genotypes at 5 days postinoculation. (C) Growth of *Pfl*-2-79 in the stated plant genotypes at 5 days postinoculation. (D) Extent of *Bgt* infection measured by determining the percentage of conidia that form a haustorium on the indicated plant lines. (E) Accumulation of *PR1* transcripts in wild-type Col-0 and Col-0 *as1-4* plants at the given times after *B. cinerea* inoculation. (F) Portions of leaves from the indicated plant genotypes stained with Aniline blue to detect callose deposition (white dots) after inoculation with *Pst*DC3000. (Scale bars, 250  $\mu$ m.) (G) Quantification of callose deposition. Error bars represent 95% confidence limits. These experiments were repeated at least three times with similar results.

**AS1 Function in Responses to Phytopathogens Is Independent of Its AS2-Associated Role in Development.** AS2, a member of the LATERAL ORGAN BOUNDARIES (LOB) family of plant-specific proteins, forms heterodimers with AS1 and is likely to function in the same developmental process (9, 10). The *as2* and *as1* lines have similar morphological phenotypes (10), but whereas *as1* plants were more resistant to *B. cinerea*, *as2* mutants were as susceptible as wild type (Fig. 4A). Furthermore, analysis of the weak *as1-13* allele, which shows only mild morphological defects, revealed that this line still exhibited significant protection against *B. cinerea* (Fig. 4A). These findings suggest, firstly, that the





**Fig. 4.** *AS1* is an evolutionarily conserved regulator of plant immunity that functions independently of *AS2* and *KN1*. (A) The given *Arabidopsis* genotypes were spray-inoculated with a *B. cinerea* spore suspension. The *35S:GR* and *35S:KN1-GR* lines were exogenously treated with 1  $\mu$ M dexamethasone 48 h before *B. cinerea* inoculation and disease symptoms scored at 96 hpi by using a disease index. (B and C) Wild-type *A. majus* (B) and *phan* (C) plants were challenged with *B. cinerea* and scored at 96 hpi. (D) Quantification of *B. cinerea* disease phenotypes in the stated plant genotypes by using a disease index. (E) Wild-type (empty bars) and *phan* (filled bars) *A. majus* plants were inoculated with either *PstDC3000* or *Pfl2-79*, and bacterial growth was determined at 5 days after inoculation. (F and G) Wild-type *N. sylvestris* plants (F) and an *N. sylvestris* antisense line (G) depleted in *NSPHAN* RNA were inoculated with *B. cinerea* and scored at 96 hpi. (H) Quantification of *B. cinerea* disease phenotypes in the stated plant genotypes. (I) Wild-type *N. sylvestris* (empty bars) and an *N. sylvestris* antisense line depleted in *NSPHAN* RNA (*nsphan*) (filled bars) were challenged with either *PstDC3000* or *Pfl2-79* and bacterial growth was determined 5 days after inoculation. Error bars represent 95% confidence limits. These experiments were repeated three times with similar results.

resistance effects of *as1* mutations are not due to altered morphology and, secondly, that the role of *AS1* in repressing JA-induced defense genes is separate from its *AS2*-dependent role in development.

The only recognized developmental function of *AS1* and *AS2* is to prevent expression of *KNOX* genes (1, 10), and misexpression of the *KNOX* gene *KN1* is sufficient to cause lobed leaves similar to those of *as1* or *as2* (11). Because the roles of *AS1* in development and defense could be separated by their dependence on *AS2*, we predicted that *KN1* misexpression would not cause increased resistance to *B. cinerea*. To test this, we induced ectopic *KNOX* activity by using a fusion of *KN1* to the rat glucocorticoid receptor under control of the constitutive *35S* promoter. This activity causes rapid dexamethasone-dependent changes in gibberellin and cytokinin levels and development of *as1*-like lobed leaves (11). Inducing *KN1* activity before *B. cinerea* inoculation reduced the expression of a *KN1* target gene, which encoded a gibberellin 20-oxidase (11) (data not shown) but did not confer increased resistance to *B. cinerea* (Fig. 4A). Constitutive expression of another *KNOX* gene, *BREVIPEDICELLUS* (*BP*) (40), that is an endogenous target of *AS1* and *AS2*, gave a similar result. We also tested potential involvement of the *ERECTA* receptor-like kinase, which has multiple developmental roles mainly associated with cell growth. Although *er* mutations enhance the *as1* and *as2* mutant phenotype (10) and cause increased susceptibility to some bacterial pathogens (41), *er* mutants showed the same response to *B. cinerea* as wild type (Fig. 4A).

**The Defense Function of *AS1* Is Evolutionarily Conserved.** *AS1* is orthologous to *PHAN* in *Antirrhinum* (2) and *NSPHAN* in tobacco (4), which have conserved roles in repressing *KNOX* genes in leaves. We therefore determined whether the functions of *AS1* in plant–pathogen interactions were also conserved. Wild-type *A. majus* showed strong disease development 10 days after spraying with a suspension of *B. cinerea* spores, whereas *phan* mutants had significantly reduced levels of disease (Fig. 4B–D). Conversely, *phan* mutants supported an increase in *PstDC3000* and *Pfl2-79* growth relative to wild type (Fig. 4E). Similarly, an antisense *N. sylvestris* line depleted in *NSPHAN* RNA (4) showed reduced lesion development in response to *B. cinerea* relative to wild type (Fig. 4F–H) and increased *PstDC3000* and *Pfl2-79* growth (Fig. 4I). Collectively, these data suggest that *AS1* has an evolutionarily conserved role in plant–pathogen interactions.

## Discussion

We have uncovered an unexpected role for the developmental regulator, *AS1*, in plant–pathogen interactions. Loss-of-function mutations in this gene convey strikingly increased resistance against both *B. cinerea* and *A. brassicicola*. Therefore, *AS1* may repress the inducible defense response against a relatively broad range of necrotrophic fungi. Our findings suggest that *AS1* acts to suppress resistance to necrotrophs by repressing the expression of JA-induced resistance proteins, including *THI1*, which when overexpressed is sufficient to confer enhanced resistance to the necrotrophic pathogen *Fusarium oxysporum* in *Arabidopsis* (42). Because *AS1* associates physically with the promoters of JA-induced genes, this repression is likely to be direct. Target gene expression and increased resistance, however, remain dependent on functional JA signaling in the absence of *AS1* activity. For at least one target gene, the promoter region occupied by *AS1* is distinct from that required for response to JA, and *AS1* expression is not affected when JA signaling is disrupted. This observation suggests that induction by JA signaling and repression by *AS1* act in parallel, converging on target gene promoters. One rationale for the repressive role of *AS1* is that it damps the JA response, preventing premature deployment of the immunity response and its associated costs.



