RXLR Effector AVR2 Up-Regulates a Brassinosteroid-Responsive bHLH Transcription Factor to Suppress Immunity

Dionne Turnbull, Lina Yang, Shaista Naqvi, Susan Breen, Lydia Welsh, Jennifer Stephens, Jenny Morris, Petra C. Boevink, Pete E. Hedley, Jiasui Zhan, Paul R. J. Birch, and Eleanor M. Gilroy*

Cell and Molecular Science, James Hutton Institute (D.T., L.Y., S.N., L.W., J.S., J.M., P.C.B., P.E.H., P.R.J.B., E.M.G.), and Division of Plant Science, School of Life Science (at The James Hutton Institute) (D.T., L.Y., S.N., P.R.J.B.), University of Dundee Invergowrie, Dundee, DD2 5DA, United Kingdom; Fujian Key Laboratory of Plant Virology, Institute of Plant Virology, Fujian Agricultural and Forestry University, Fuzhou, Fujian 350002, China (L.Y., J.Z.); James Hutton Ltd (at The James Hutton Institute), Plant Sciences Division, Research School of Biology, The Australian National University, Canberra 2601, Australia (S.B.); and Effector Consortium, James Hutton Institute, Invergowrie, Dundee DD2 5DA, United Kingdom (D.T., L.Y., S.N., L.W., P.C.B., P.E.H., P.R.J.B., E.M.G.)

ORCID IDs: 0000-0002-5301-4268 (E.M.G.); 0000-0002-5761-9782 (J.S.); 0000-0001-5232-9973 (S.B.).

An emerging area in plant research focuses on antagonism between regulatory systems governing growth and immunity. Such cross talk represents a point of vulnerability for pathogens to exploit. AVR2, an RXLR effector secreted by the potato blight pathogen Phytophthora infestans, interacts with potato BSL1, a putative phosphatase implicated in growth-promoting brassinosteroid (BR) hormone signaling. Transgenic potato (Solanum tuberosum) plants expressing the effector exhibit transcriptional and phenotypic hallmarks of overactive BR signaling and show enhanced susceptibility to P. infestans. Microarray analysis was used to identify a set of BR-responsive marker genes in potato, all of which are constitutively expressed to BR-induced levels in AVR2 transgenic lines. One of these genes was a bHLH transcription factor, designated STCHL1, homologous to AICIB1 and A1HBI1, which are known to facilitate antagonism between BR and immune responses.Transient expression of either AVR2 or CHL1 enhanced leaf colonization by P. infestans and compromised immune cell death activated by perception of the elicitin Infestin1 (INF1). Knockdown of CHL1 transcript using Virus-Induced Gene Silencing (VIGS) reduced colonization of P. infestans on Nicotiana benthamiana. Moreover, the ability of AVR2 to suppress INF1-triggered cell death was attenuated in NbCHL1-silenced plants, indicating that NbCHL1 was important for this effector activity. Thus, AVR2 exploits cross talk between BR signaling and innate immunity in Solanum species, representing a novel, indirect mode of innate immune suppression by a filamentous pathogen effector.

Plants are sedentary and cannot escape the challenges they sense in their environment. To best utilize the available resources, plants are equipped with a large number of receptor-like proteins linked to complex networks of interacting signal transduction pathways that allow them to respond appropriately and rapidly to environmental conditions. Plants can detect a multitude of potential invaders, including bacteria, fungi, and oomycetes, and have evolved two key inducible mechanisms by which they can defend themselves. In the first instance, Microbe- or Pathogen-Associated Molecular Patterns (MAMPs/PAMPs) can be detected by Pattern Recognition Receptors (PRRs). PRRs initiate Pattern-Triggered Immunity (PTI) and provide broad-spectrum disease resistance, often to whole classes of micro-organisms (Jones and Dangl, 2006). Many pathogens deliver “effector,” specialized proteins that act outside or within plant cells to suppress immunity or modify other host processes to increase disease potential. Effectors in turn may be recognized by corresponding resistance (R) proteins, activating a rapid immune response known as effector triggered immunity, which frequently results in a localized cell death known as the hypersensitive response (HR; Jones and Dangl, 2006; Feechan et al., 2015).

Although there is some evidence in the literature indicating that growth and defense can be regulated simultaneously (Francisco et al., 2016; Campos et al., 2016; de Wit et al., 2013), much of the research done in the area of growth and immunity has shown that activation of plant defense responses requires a major reallocation of resources away from growth to immunity (Huot et al., 2014). Consequently, plants must tightly regulate and fine-tune the signals that control this trade-off. This compromise between growth and defense is controlled at multiple levels and shown to depend on the action of several plant hormones, including jasmonates, gibberellins, brassinosteroids (BR), and salicylic acid (Albrecht et al., 2012; Belkhadir et al., 2012; Yang et al., 2013; Lozano-Durán et al., 2013;
Chandran et al., 2014; Wang and Wang, 2014; Fan et al., 2014; Malinovsky et al., 2014). Recent work has particularly focused on antagonistic cross talk between the BR signaling pathway, involved in growth and development, and aspects of plant immunity (Jiménez-Góngora et al., 2015; Albrecht et al., 2012; Belkhadir et al., 2012). The BR pathway is well characterized in Arabidopsis thaliana and is essential in growth and development; regulating cell expansion, vascular differentiation, etiolation, and reproductive development. Consequently, plants insensitive to BR or unable to generate BRs are acutely dwarfed, exhibiting small, dark green leaves with severe defects in cell division and elongation (Zhipnova et al., 2013).

The current understanding of the Arabidopsis BR pathway is potentially oversimplified, as every regulatory step may involve a number of closely related but less well-characterized family members, paralogues, and splice variants (Mora-García et al., 2004; Maselli et al., 2014; Wang and Mao, 2014; Zhang et al., 2014). In the current model, BRs bind directly the LRR-receptor like kinase BR Insensitive 1 (BRI1; Li and Chory, 1997). This induces BRI1 dimerization, hetero-oligomerization with BRI1-Associated Kinase 1 (BAK1), and release of the negative regulators BRI1 Kinase Inhibitor (BKK1) and Botrytis-Induced Kinase 1 (BKI1) (Lozano-Durán and Zipfel, 2015; Heese et al., 2007; Nam and Li, 2002). BRI1 activity causes successive phosphorylation and activation of the receptor-like cytoplasmic kinases BR Signaling Kinases (BSKs) and Constitutive Differential Growth 1 (CDG1; Kim et al., 2009, 2011; Tang et al., 2008; Sreeramulu et al., 2013) followed by interaction with BRI1 Suppressor 1 (BSU1). BIN2 inactivation allows the accumulation of the transcription factors (TFs) Brassinazole-Resistant 1 (BZR1) and BRI1-Ems Suppressor 1 (BES1). Upon accumulation, the TFs BZR1 and BES1 undergo dephosphorylation by the protein phosphatase PP2A, which allows them to be relocated to the nucleus where they orchestrate the expression of BR-responsive genes (Kim and Wang, 2010). Previously reported transcriptional changes include the up-regulation of expansins and cell wall modifying genes, regulation of other plant hormone pathways, and light signaling (Müssig et al., 2002; Goda et al., 2002).

Perception of BR has been shown to suppress PTI, initially hypothesized to be the result of competition for BAK1 between competing LRR-receptor like kinases, the flagellin recognizing receptor, FLS2, and BR receptor, BRI1 (Belkhadir et al., 2012). However, Albrecht et al. (2012) showed that BR perception had no effect on the amount of BAK1 available for interaction with FLS2. Furthermore, while BAK1 has been shown to play no role in chitin perception (Schwessinger et al., 2011), treatment with exogenous BR inhibited the ROS burst associated with chitin perception by Chitin Elicitor Receptor Kinase 1 (CERK1), indicating that the link between BR perception and immune signaling is not solely due to this shared coreceptor (Albrecht et al., 2012). More evidence points toward conflict between transcriptional regulators of both pathways. Recently the interaction and phosphorylation of BES1 by the PTI-activated MAPK, MPK6, has been identified as a possible mechanism of PTI-induced inhibition of BR signaling (Kang et al., 2015). Furthermore, BZR1 has been demonstrated to be a central regulatory component in the cross-talk between growth and immunity (Lozano-Durán et al., 2013). BZR1 is proposed to be linked to the BR-dependent induction of expression of the basic-Helix-Loop-Helix (bHLH) TFs, Cryptochrome-Interacting Basic-Helix-Loop-Helix 1 (CIB1), BR Enhanced Expression 2 (BEE2) Homolog of BEE2 Interacting with IBH 1 (HBII1), and that act partially as negative regulators of PTI in Arabidopsis (Lozano-Durán et al., 2013; Malinovsky et al., 2014). AtHBII1 has been best characterized, acting as both a positive regulator of BR responses (Bai et al., 2012) and a negative regulator of immunity (Fan et al., 2014).

A broad range of host targets and activities has been elucidated for pathogen effectors secreted into host plant cells. Many effectors act on positive regulators of immunity to inhibit their activity (Whisson et al., 2016; Deslandes and Rivas, 2012; Dou and Zhou, 2012; Feng et al., 2012; Block and Alfano, 2011). In contrast, a number of pathogen effectors have been found to target host proteins that negatively regulate immunity (Yang et al., 2016; Boevink et al., 2016a; Wang et al., 2015; Cui et al., 2010; Chen et al., 2010). Negative regulators in the host that are required by pathogens to aid disease progression, and are thus manipulated by effectors to promote or use their activity, have been designated as susceptibility (S) factors (van Schie and Takken, 2014; Boevink et al., 2016b).

The cytoplasmic RXLR-EER effector from Phytophthora infestans, AVR2, accumulates in the pathogen at the site of haustorium formation, is up-regulated during the biotrophic phase of infection on potato (Solanum tuberosum), and is recognized inside plant cells by the...
host resistance protein R2 (Gilroy et al., 2011a). A kelch-repeat containing phosphatase, StBSL1 (BSU1-Like 1), was identified as an interactor of AVR2 in potato. Although silencing of BSU1 in Nicotiana benthamiana perturbed recognition of AVR2 by R2 family members, there was no apparent developmental phenotype or impact on susceptibility to P. infestans (Saunders et al., 2012). StBSL1 is homologous to one of the four members of the BSU1 family known in Arabidopsis. Interestingly, the knockout mutants of BSU1 or BSL1 in Arabidopsis are also phenotype neutral (Mora-García et al., 2004). Most evidence of the role of this phosphatase family in the BR pathway has been generated by studying BSU1, which is weakly expressed in mature leaves and has recently been shown to be a Brassicaceae-specific family member (Mora-García et al., 2004; Maselli et al., 2014).

To investigate the role of pathogen effector AVR2 in late blight development, we generated transgenic potato plants that stably express this effector. We observed that AVR2 transgenic lines exhibited developmental and transcriptional changes that are hallmarks of BR pathway activation, and showed enhanced susceptibility to P. infestans.

One transcript (bHLH7) up-regulated by BR treatment, and constitutively expressed in AVR2 lines, was of particular interest as it shares homology with TFs AtCIB1 and AtHBI1, shown to regulate cross talk between PTI and BR signaling (Fan et al., 2014; Malinovsky et al., 2014; Bai et al., 2012). We utilized Agrobacterium-mediated transient expression of AVR2 and StCIB1/HBI1-like1 (StCHL1) to assess their impact on susceptibility of N. benthamiana to P. infestans and the BAK1-dependent responses to the oomycete PAMP elicitor Infestin 1 (INF1; Du et al., 2015; Derevnina et al., 2016). We further evaluated the role of NbCHL1 by Virus Induced Gene Silencing (VIGS) in N. benthamiana and demonstrated that the ability of AVR2 to suppress INF1-triggered cell death is at least partially dependent on CHL1.

RESULTS AND DISCUSSION

Transgenic Potato Lines Expressing AVR2 Display BR-Associated Developmental Phenotypes

Transgenic potato cv Desiree lines were generated with constitutive, 35S-promoter-driven AVR2 expression. Two lines were taken forward for further analysis.
Figure 2. Microarray analysis of BR (EBL treatment) response in potato cv Desiree. A, Microarray validation by quantitative real-time PCR (qRT-PCR) of two independent biological replicates plotted in one graph. Fold-change from microarray data plotted against fold-change from qRT-PCR for five selected BR marker genes examined at 24 hpt. Fold-change log2 transformed to allow symmetry of up- and down-regulation. Linear regression was used to determine a coefficient of determination (R^2). B, Table of selected marker genes showing significant differential expression with BR treatment. Fold-change values are shown from the microarray data, qRT-PCR validation, and an independent biological replicate. C, Relative expression of BR-regulated genes in untreated potato cv Desiree (wild type [WT]; given a value of 1), wild type at 24 h after treatment with EBL, and constitutive levels of expression in 35S:AVR2 potato plants, assessed by qRT-PCR. Expression was normalized to StUb1 and shown relative to wild-type untreated plants. Graph shows the average of three technical replicates ± SD, with similar trend observed in two independent biological replicates.
(#29, #39) to investigate the impact of AVR2 on BR signaling. Critically, in contrast to untransformed cv Desiree, when the resistance gene R2 was transiently expressed in leaves, both AVR2 transgenic lines responded with a clear HR confirming the presence of a recognized AVR2 protein (Supplemental Fig. S1). 35S:AVR2 plants displayed a variety of developmental phenotypes including twisted stems with curled leaflets (Fig. 1, A and B). Compound leaf formation was also affected, with reduced numbers of leaflets, and organ fusion where leaflets failed to separate was evident. Additionally, petioles and leaves exhibited loss of symmetry and tended to extend from the main stem in a curved manner (Fig. 1, A and B). These phenotypes are reminiscent of BR-overactive phenotypes in Arabidopsis generated by BRI1 and DWF4 overexpression (Wang et al., 2001), bik1 mutation (Lin et al., 2013), and BZR1 overexpression (Gendron et al., 2012). Visualizing 35S:AVR2 potato leaves under the microscope revealed increased epidermal cell size (Supplemental Fig. S2). Additionally, stomatal frequency was found to be significantly decreased (Fig. 1, C and D), consistent with the decreased stomatal frequency observed when BSL family members are overexpressed in Arabidopsis (Kim et al., 2012). This evidence suggests that AVR2 may activate BR responses in potato. BR-responsive gene expression markers were next sought to confirm this.

**BR-Responsive Genes Are Up-Regulated in AVR2-Expressing Potatoes**

To understand the impact of BR signaling on the potato transcriptome and to identify a set of BR marker genes relevant to this crop species, microarray analysis of BR-treated potato cv Desiree was performed. The top 50 differentially expressed genes at 3 and 24 h after
foliar spray with the BR epibrassinolide (EBL) compared to water-sprayed controls are shown in Supplemental Tables S1 to S4. Five marker genes were selected from the microarray dataset (four up-regulated and one down-regulated), validated by quantitative real time PCR (qRT-PCR) and observed to be similarly differentially expressed following EBL treatment in independent biological replicates (Fig. 2A). Among these were 3 of the 50 most highly induced genes following EBL treatment (Supplemental Table S1): CAB50, encoding a chlorophyll a-b binding protein associated with light harvesting; P69F, encoding a subtilisin-like proteinase; and a basic helix-loop-helix TF (bHLH7) with significant blast hit matches to two closely related bHLH TFs, CIB1 and HBI1 from Arabidopsis (Fig. 2B; Supplemental Figs. S3 and S4). The potato bHLH7 sequence was thus renamed StCHL1. AtCIB1 and AtHBI1 are known to play a role in cell elongation and responses to BR (Bai et al., 2012). Another sequence up-regulated by 2- to 7-fold by BR treatment had high sequence homology to a BR-inducible member (SAUR67) of the SAUR (Small auxin upregulated RNA) family (Spartz et al., 2012). Gibberellin-2-Oxidase 1 (StGAOx1), an enzyme involved in catalysis, or breakdown of endogenous GA in plants (Lo et al., 2008; Bai et al., 2012) was down-regulated around 5-fold by BR in this microarray. We also designed qRT-PCR primers to a candidate potato ortholog (reciprocal best BLAST hit) of a known BR-up-regulated marker gene EXP8 in Arabidopsis (Malinovsky et al., 2014; Bai et al., 2012), StEXP8, which was not detected as significantly up-regulated in our microarrays, was confirmed to be up-regulated using qRT-PCR. These six markers were used to assess BR pathway activity in 35S:AVR2 potato lines #29 and #39. Increased transcript accumulation of the marker genes CAB50, P69F, StCHL1, StSAUR67, and StEXP8 following EBL treatment in untransformed potato reached levels similar to their constitutive expression in 35S:AVR2 potato lines, whereas EBL treatment down-regulated StGAox1 to levels similar to those in untreated 35S: AVR2 potato lines (Fig. 2C). This supports the phenotypic observations that these AVR2 lines display constitutive BR pathway overactivity. Future microarray analyses will reveal the global transcriptional changes resulting from AVR2 expression in these transgenic lines.

PTI Down-Regulates BR-Responsive Genes in Potato

The cross talk between PTI and BR identified in Arabidopsis would predict that our BR response marker genes in potato should also be down-regulated by PAMP treatments (Jiménez-Góngora et al., 2015). Consequently, the expression of BR-responsive genes was examined by qRT-PCR following treatment of wild-type potato cv Desiree with the bacterial flagellin-derived PAMP flg22 and with P. infestans culture filtrate (CF), which can be regarded as a cocktail of P. infestans PAMPs (McLellan et al., 2013). As anticipated, in contrast to the PTI marker genes StWRKY7 and StACRE31 (McLellan et al., 2013; Nguyen et al., 2010), the BR marker genes were actively down-regulated following flg22 and CF treatments, as were additional genes, StDWF5 and StSTDH, associated with BR biosynthesis (Fig. 3, A and B). Recently, AtHBI1 has been implicated as a cross talk regulator between PTI and BR-associated growth and may act redundantly with CIB1 and BEE2, all of which are down-regulated by PAMPs in Arabidopsis (Malinovsky et al., 2014; Fan et al., 2014, Bai et al., 2012). Our finding supports the existing

Figure 4. AVR2 expression in potato results in increased susceptibility to P. infestans. A, Lesion size of transgenic P. infestans isolate 88069 expressing tdTomato (McLellan et al., 2013; diameter in mm) on 35S:AVR2 potato at 7 d postinoculation of sporangia suspension. Data shown combines two independent replicates, each comprising 10 or more leaves per plant line, taken from three or more individual plants, with two inoculations per leaf. Error bars represent SEM; letters denote significant difference (P < 0.001, one-way ANOVA with Holm-Sidak). B, Representative leaf images showing increased lesion size of transgenic tdTomato expressing P. infestans isolate 88069 (McLellan et al., 2013) on 35S:AVR2 potato compared to untransformed wild-type (WT) potato. Images are taken under UV light.
observation that BR-responsive genes are downregulated during PTI in Arabidopsis (Jiménez-Góngora et al., 2015) by demonstrating that this antagonism also occurs in the Solanaceae.

AVR2 Transgenic Plants Are More Susceptible to P. infestans

To further investigate the impact of AVR2 expression in potato, transgenic 35S:AVR2 lines and wild-type Desiree controls, were inoculated with a concentrated suspension of P. infestans sporangia. At 7 d after inoculation, a significant increase was observed in P. infestans lesion size on leaves of the AVR2 transgenic lines, compared to the wild-type potato (Fig. 4, A and B), indicating that these plants, in addition to exhibiting phenotypes of BR overactivity and constitutive expression of BR-responsive marker genes, are also more susceptible to late blight.

Expression of AVR2 Suppresses INF1-Triggered Cell Death and Enhances P. infestans Colonization in N. benthamiana

As expression of AVR2 in potato increased susceptibility, we predicted that transient expression in N. benthamiana would also enhance P. infestans colonization. N. benthamiana is a model solanaceous host for P. infestans that has been extensively used in RXLR effector research as it facilitates transient expression, cell biology, and silencing studies (Whisson et al., 2016). AVR2 was transiently expressed using Agrobacterium, infiltration sites were subsequently inoculated with P. infestans sporangia, and disease progression monitored. Transient AVR2 expression resulted in significantly increased lesion sizes (Fig. 5, A and B). This observation is consistent with the stable expression of AVR2 in potato, which also enhanced P. infestans leaf colonization (Fig. 4, A and B) confirming that AVR2 enhances late blight susceptibility in both plant systems.

A major virulence strategy of pathogens is to suppress aspects of plant immunity, impairing host defenses to increase the chance of a successful infection. INF1 is an oomycete elicin that is recognized as a PAMP by PRRs in N. benthamiana and a number of Solanum species, triggering an immune response that results in localized cell death in a BAK1-dependent manner (Du et al., 2015; Chaparro-Garcia et al., 2011). This can be used as a phenotypic read-out for effective PTI in N. benthamiana and is already known to be suppressed by P. infestans effector AVR3a (Bos et al., 2010). Agrobacterium-expressing INF1 was coinfiltred with Agrobacterium expressing AVR2 and AVR3a as a positive control. Expression of AVR2 suppressed INF1-mediated cell death to a similar extent as the AVR3a control (Fig. 5, C and D). These results demonstrate that AVR2 plays a role in attenuating plant immunity. This is a key role of pathogen effectors and a crucial part of the zig-zag model of molecular plant-pathogen interactions (Jones and Dangl, 2006).

Figure 5. AVR2 negatively regulates immunity to P. infestans and suppresses INF1 cell death. A, Average lesion size (diameter) of P. infestans 88069 colonization on N. benthamiana, inoculated 24 h after Agrobacterium-mediated transient expression of GFP-AVR2 or empty vector (EV) control. Results combine three biological reps, consisting of at least six plants each with six infiltrations per construct. Error bars show SEM; letters denote significant difference (P ≤ 0.001) using one-way ANOVA (Holm-Sidak). B, Representative leaf images showing increased P. infestans 88069 lesion size with GFP-AVR2 expression. Images were taken under UV light to show full extent of infection. C, Percentage of inoculation sites leading to cell death following coexpression of INF1 with AVR2, AVR3a (positive control) or an empty vector (negative control) in N. benthamiana. Error bars show SEM; letters denote significant difference (P ≤ 0.001) using one-way ANOVA (Holm-Sidak). Results combine at least three experimental replicates, consisting of four or more plants, with three or more infiltrations per plant, per combination. D, Representative leaf image showing suppression of INF1-triggered cell death when AVR2, or AVR3a are coexpressed.

Transient Overexpression of StCHL1 Suppresses INF1-Triggered Cell Death and Enhances P. infestans Colonization in N. benthamiana

Of the BR-induced genes that are constitutively up-regulated in 35S:AVR2 transgenic potato lines, StCHL1 (bHLH7) was of particular interest as a homolog of bHLH domain containing proteins shown to regulate cross talk between growth and immunity in Arabidopsis (Fan et al., 2014; Malinovsky et al., 2014; Suplemental Figs. S3 and S4). StCHL1 was one of the most strongly up-regulated genes following BR treatment of potato, with expression 13-fold higher
than untreated plants (Fig. 2C). StCHL1 was thus taken forward for functional analysis to determine any impact on immunity in solanaceous plants. Transient coexpression of *Agrobacterium* delivered StCHL1 (Supplemental Fig. S5) with the PAMP INF1 resulted in suppression of INF1-triggered cell death, indicating compromised PTI (Fig. 6, A and B). In addition, transient expression of StCHL1 attenuated the induction of the PTI marker genes *NbWRKY7* and *NbACRE31* upon treatment with *P. infestans* CF, further demonstrating that StCHL1 antagonizes immunity. AVR2 expression also negatively affected PTI marker gene induction in a similar manner (Fig. 6C). Finally, when StCHL1 was transiently overexpressed, *P. infestans* leaf colonization of *N. benthamiana* was significantly enhanced, consistent with previous reports that its homologs HBI1 and CIB1 act as negative regulators of immunity (Malinovsky et al., 2014; Fan et al., 2014; Fig. 6, D and E). These results suggest that StCHL1, like Arabidopsis CIB1 and HBI1 TFs, represents an important node of cross talk between BR signaling and PTI in solanaceous plants, acting to suppress the latter.

**VIGS of NbCHL1 in N. benthamiana Reduces *P. infestans* Colonization and Attenuates AVR2 Suppression of INF1-Triggered Cell Death**

We identified the *N. benthamiana* ortholog of StCHL1 (Supplemental Figs S3 and S4) and designed two VIGS constructs (5' and 3') to silence it (Supplemental Fig. S6A). Expression of NbCHL1 VIGS constructs in *N. benthamiana* resulted in dwarfed plants with some mildly curled leaves reminiscent of plants with perturbed BR signaling (Supplemental Fig. S6B). NbCHL1

**Figure 6.** StCHL1 suppresses immunity and increases susceptibility to *P. infestans* in *N. benthamiana*. A, Graph shows percentage of leaf infiltration sites at 5 dpi resulting in cell death following *Agrobacterium*-mediated coexpression of INF1 with either StCHL1 or an empty vector (EV) control. Error bars show SEM, a ≠ b (*P* < 0.001) in one-way ANOVA (Holm-Sidak). Results are combined from four experimental replicates consisting of at least four plants, each, with at least six infiltrations per plant per expression combination. B, Representative leaf image showing suppression of INF1 cell death when CHL1 is coexpressed. C, StCHL1 or an empty vector control were transiently expressed in *N. benthamiana*. Sites were inoculated with *P. infestans* 88069 sporangia suspension 24 h later, with lesions measured (diameter in mm) at 7 dpi. Error bars show SEM; letters denote significant difference (*P* < 0.001 in one-way ANOVA, Holm-Sidak). Results are combined from four experimental replicates. D, Representative leaf image showing increased *P. infestans* colonization following StCHL1 expression in *N. benthamiana*. Sites were inoculated with *P. infestans* 88069 sporangia suspension 24 h later, with lesions measured (diameter in mm) at 7 dpi. Error bars show SEM; letters denote significant difference (*P* < 0.001 in one-way ANOVA, Holm-Sidak). Results are combined from four experimental replicates. E, PTI marker gene (*NbWRKY7, NbACRE31*) expression in *N. benthamiana* 1 h after *P. infestans* CF treatment, relative to untreated plants (which were given a value of 1) by qRT-PCR. Treatment occurred 2 d after *Agrobacterium*-mediated transient expression of AVR2, StCHL1, or an empty vector control. Expression levels were normalized to *NbEF1a*. Data represents the average of three technical replicates combined, ±SED.
transcript was reduced by 40% to 60% in three biological replicates of plants expressing either VIGS construct (Supplemental Fig. S6C). We inoculated detached leaves from NbCHL1 (5’ and 3’) silenced plants with P. infestans sporangia, and disease progression was monitored for 6 to 7 d in four biological replicates. Silencing of NbCHL1 caused a significant decrease of P. infestans colonization measured by both sporangia counting and lesion diameter (Fig. 7, A and B). Critically, we observed a significant reduction in the ability of AVR2 to suppress INF1-triggered cell death in the NbCHL1 VIGS plants, whereas suppression by the control effector AVR3a was unaltered. The failure to completely attenuate cell death suppression by AVR2 could be due to the low silencing efficiency of the VIGS constructs.
A common mode-of-action for phytopathogen effectors is to reduce or inhibit activity of their host targets (Rovenich et al., 2014). The enhanced *P. infestans* pathogenicity facilitated by AVR2 expression, in combination with its suppressive effect on PTI, does not support a model in which AVR2 inhibits the BR pathway. Indeed, it demonstrates the opposite to be true; the pathogen benefits from the role of effector AVR2 in activating the BR pathway, resulting in the up-regulation of CHL1 to act as a negative regulator of immunity (Fig. 8). We predict that if assays in this paper were repeated with known activators of the BR pathway, for example, the GSK3 inhibitor, Bikinin (De Rybel et al., 2009), the effect on INF1 cell death, *P. infestans* growth, and BR marker gene expression would be similar to expression of AVR2.

A number of examples highlight that the presence and activity of host effector targets can be required for host susceptibility (Yang et al., 2016; Boevink et al., 2016a; Wang et al., 2015; Cui et al., 2010; Chen et al., 2010). Plant genes that are required to support infection, often acting as negative regulators of immunity, are known as susceptibility (S) factors (van Schie and Takken, 2014; Boevink et al., 2016b). The mutation or knockdown of S factors limits the ability of the pathogen to cause disease. When S factors are direct effector targets, their presence is required for manipulation by effector activity to promote disease. Here, silencing of *NbCHL1* compromised susceptibility of *N. benthamiana* to *P. infestans* and the ability of AVR2 to suppress INF1-triggered cell death, implying it should be considered an S factor and could be an indirect target of AVR2 activity to increase host susceptibility (van Schie and Takken, 2014).

In conclusion, this work identifies AVR2 as the first effector from a filamentous plant pathogen to exploit the antagonistic cross talk between BR signaling and innate immunity. This represents a novel, indirect mode of innate immune suppression by a pathogen effector. Future work will focus on the mechanism by which AVR2 promotes BR pathway activity to the benefit of the pathogen and examine how this activity is perceived by R2-like NB-LRRs. In particular, given that AVR2 promotes the BR pathway, future work will determine the precise relationship between this effector and its target, the candidate phosphatase BSL1, which can be regarded as a likely positive regulator of BR signaling (Fig. 8). The intricacies of cross-talk between growth and innate immunity in plants raise a crucial point for breeding efforts: a push toward one may be at the expense of the other. This highlights a need for balance and to maintain a whole-plant view toward optimizing both yield and disease resistance in our agricultural systems.

**MATERIALS AND METHODS**

**Agrobacterium-Mediated Transient Expression**

Constructs used in this work were transformed by electroporation into the *Agrobacterium* strain AGL1 VirG pSOLUP. Liquid YEB were inoculated using bacteria from fresh plates and incubated overnight at 28°C with shaking. Cultures were spun down at 3,000 rpm for 10 min, and the bacterial pellet resuspended in 10 mM MES 10 mM MgCl2 buffer. OD600 was adjusted to 0.5 for cell death assays, or to 0.1 for *Phytophthora infestans* colonization assays, with acesulfame added at 200 μM. Leaves of *Nicotiana benthamiana* or potato (*Solanum tuberosum*) cv Desiree were infiltrated on the abaxial surface, using a 1-ml syringe after wounding with a needle. INF1 cell death suppression assays were performed as previously described (Gilroy et al., 2011b).

**P. infestans Colonization**

*P. infestans* strain 88069 expressing tdTomato fluorescent protein (McLellan et al., 2013, Saunders et al., 2012) was grown on rye (*Secale cereale*) agar supplemented with 20 μg/ml geneticin (Thermo Fisher Scientific Ltd.) as a selective antibiotic. To harvest sporangia, 10-d-old plates were flooded with 5 ml sterile distilled water before scraping with a spreader onto a 70-μm cell strainer placed on a 50-ml Falcon tube. The suspension containing sporangia was spun down at 3,000 rpm for 10 min, after discarding supernatant, the pellet was resuspended in sterile distilled water. Sporangia were quantified using a hemocytometer, and adjusted to a concentration of 50,000 mL⁻¹. Then 10-μl droplets were pipetted onto the abaxial surface of detached leaves, maintained in sealed boxes with moist tissue. Boxes were kept in darkness for the first 24 h to reduce UV degradation of sporangia. Lesions were measured at the widest point 7 d postinfiltration. When used in combination, *P. infestans* was inoculated 24 h after infiltration with *Agrobacterium* suspension. Lesions were measured at the widest point 7 d postinoculation. Disease scoring data and INF1-mediated cell death suppression assay data (see above) were subjected to

![Figure 8. Proposed model indicating how AVR2 tips the balance between growth and immunity to promote potato late blight disease.](image-url)

Perception of BR by the receptor BRI1 activates the BR signaling pathway, inducing CHL1 (black arrows), which is proposed to serve as a positive regulator of BR signaling (Fig. 8). The intracellular role of effector AVR2 in activating the BR pathway, resulting in the up-regulation of CHL1 to act as a negative regulator of immunity (Fig. 8). We predict that if assays in this paper were repeated with known activators of the BR pathway, for example, the GSK3 inhibitor, Bikinin (De Rybel et al., 2009), the effect on INF1 cell death, *P. infestans* growth, and BR marker gene expression would be similar to expression of AVR2.

A number of examples highlight that the presence and activity of host effector targets can be required for host susceptibility (Yang et al., 2016; Boevink et al., 2016a; Wang et al., 2015; Cui et al., 2010; Chen et al., 2010). Plant genes that are required to support infection, often acting as negative regulators of immunity, are known as susceptibility (S) factors (van Schie and Takken, 2014; Boevink et al., 2016b). The mutation or knockdown of S factors limits the ability of the pathogen to cause disease. When S factors are direct effector targets, their presence is required for manipulation by effector activity to promote disease. Here, silencing of *NbCHL1* compromised susceptibility of *N. benthamiana* to *P. infestans* and the ability of AVR2 to suppress INF1-triggered cell death, implying it should be considered an S factor and could be an indirect target of AVR2 activity to increase host susceptibility (van Schie and Takken, 2014).

In conclusion, this work identifies AVR2 as the first effector from a filamentous plant pathogen to exploit the antagonistic cross talk between BR signaling and innate immunity. This represents a novel, indirect mode of innate immune suppression by a pathogen effector. Future work will focus on the mechanism by which AVR2 promotes BR pathway activity to the benefit of the pathogen and examine how this activity is perceived by R2-like NB-LRRs. In particular, given that AVR2 promotes the BR pathway, future work will determine the precise relationship between this effector and its target, the candidate phosphatase BSL1, which can be regarded as a likely positive regulator of BR signaling (Fig. 8). The intricacies of cross-talk between growth and innate immunity in plants raise a crucial point for breeding efforts: a push toward one may be at the expense of the other. This highlights a need for balance and to maintain a whole-plant view toward optimizing both yield and disease resistance in our agricultural systems.
statistical analysis using one-way ANOVA (Holm-Sidak) in Sigmaplot (Systat Software Inc.)

Western Blot

Leaf tissue samples were taken 48 h postinfiltration with Agrobacterium suspensions and immediately frozen in liquid nitrogen. Protein extraction was carried out by boiling ground leaf tissue samples in 2x SDS sample buffer with 20 mM dithiothreitol (Sigma-Aldrich Co.) at 95°C for 10 min, followed by centrifugation at 13,000 rpm for 5 min. Samples were separated on 4% to 12% Bio-Tris PAGE gels with MES buffer using an X-Blot Mini Cell (all Thermo Fisher Scientific Inc.), followed by transfer to nitrocellulose membrane (Amersham Protran premium 0.45 μm NC, GE Healthcare Life Sciences) using an X10 Blot Module (Thermo Fisher Scientific Inc.) following the manufacturers’ instructions. Membranes were then stained using Ponceau solution to visualize protein loading. Membranes were blocked in 4% milk in 1xPBST, before washing 3 x 5 min washes. A secondary incubation with anti-rabbit IgG HRP (Sigma-Aldrich Co.) at 1:5,000 was carried out for 50 min, before a further 6 x 5 min washes. Signal was detected using Amersham ECL Prime as described in the manufacturers’ instructions, on Amersham Hyperfilm ECL film (both GE Healthcare Life Sciences). Films were developed with a Compact X4 Automatic Processor (Xographic Healthcare Ltd.)

VIGS

VIGS constructs consisted of approximately 250-bp PCR fragments of the gene targeted for silencing, cloned into pBinary Tobacco Rattle Virus (TRV) vectors (Liu et al., 2002; Supplemental Fig. S6). A TRV vector expressing a fragment of GFP was used as a control (Gilroy et al., 2007), and two constructs for silencing N. benthamiana are described (Supplemental Fig. S6). To achieve transient silencing, N. benthamiana plants at the four-leaf stage were pressure infiltrated with a mixture of Agrobacterium strain AGL1 containing TRV RNA1 at a final OD₆₀₀ of 0.25, and the fragment corresponding to the gene of interest, at a final OD₆₀₀ of 0.5. The two largest leaves were infiltrated fully and viral infection allowed to develop for 2 to 3 weeks before the plants were used in experiments.

Plant Material

N. benthamiana and potato cv Desiree plants were grown in general purpose compost under long-day glasshouse conditions of 16-h light at 22°C, light intensity of 130 to 150 μE m⁻² s⁻¹ and 40% humidity unless otherwise stated. N. benthamiana was used for Agrobacterium infections/P. infestans colonization at 4 to 5 weeks old, with potato at 6 to 8 weeks old.

Generation of 35S:AVR2 Transgenic Potato cv Desiree

AGL1 Agrobacterium culture (OD₆₀₀ 0.8) containing 35S:AVR2 in pGRAB vector (Gilroy et al., 2011a) and 100 μM acetylsyringone were added to 25 mL MS30 liquid medium and cocultivated with internodes from 4-week-old potato cv Desiree. Independent shoots were screened by PCR using BAR primers to confirm the presence of the transgene. Transgenic shoots were micropropagated on MS30 medium before being transferred to the glasshouse.

Quantification of Stomata

Epidermal leaf prints were obtained by pressing leaf sections onto microscope slides covered with transparent adhesive tape treated with acetone (Nagel et al., 1994). A compound microscope was used to view the epidermal leaf prints, with number of stomata and number of epidermal cells counted per 0.5-mm² area. Multiple prints were scored, representing at least three leaves per plant across three plants or more. Stomata percentage was calculated as [(no. of stomata/ (no. of stomata + no. of epidermal cells)) * 100] as previously described (Ogaya et al., 2011). For confocal microscopy, leaf tissue was first stained with Calcofluor White (Sigma-Aldrich) for 10 min before mounting sections on a microscope slide. Images were acquired on a Zeiss 710 confocal microscope with a Zeiss Epiplan APO X20/0.6 lens using 405-nm excitation.

Hormone/PTI Elicitor Treatment

EBL (Sigma-Aldrich) was first solubilized at 20 μg in ethanol. EBL treatment was carried out by foliar spray at 50 μM in distilled water, with the addition of 0.5% Tween 20 (Sigma-Aldrich Co.). Distilled water with ethanol and Tween 20 was used as a negative control. P. infestans CF was prepared by incubation of sterile amended lima bean broth with P. infestans strain 88069, left to incubate in darkness at room temperature for 5 d before filtering the solution through 70-μm nylon mesh (BioDesign CellMicroSieves; Fisher Scientific UK) to remove mycelium. Culture filtrate was filter sterilized through a 0.20-μm syringe filter (Millipore). This was used to pressure infiltrate leaves of potato cv Desiree, or N. benthamiana by wounding lightly with a needle before infiltrating with a 1-ml syringe. Uninoculated amended lima bean broth was used as a control. Flg22 peptide (Peptide Protein Research Ltd.) was dissolved at 40 μM in sterile distilled water before infiltration of leaves in the same manner.

Gene Expression Analysis

RNA was isolated from plant tissue with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturers’ instructions, including the on-column DNase treatment. RNA was quantified using a Nanodrop 1000 (Thermo Scientific) and cDNA synthesized using Superscript II (Qiagen) with oligo dT primers (Eurofins MWG Operon). qRT-PCR was performed using Maxima SYBR green qPCR Mastermix (Thermo Scientific). Detection and data acquisition were achieved with a Chromo4 real-time detector with MJ Research PTC-200 thermal cycler and Opticon Monitor 3.1.32 software (all Bio-Rad Laboratories Inc.). Reactions were incubated at 95°C for 15 min before 40 cycles of 95°C for 15 s and 60°C for 1 min, and plate reading. A melting curve was added between 58°C and 95°C, with plate read every 1°C and hold for 5 s. Data were analyzed using the Delta-ΔCt method (McElroy et al., 2013) with expression normalized to a housekeeping gene (ubiquitin for potato or Elongation Factor 1a for N. benthamiana). All primers (Eurofins MWG operon) are shown in Supplemental Table S5. Primer design was based on sequence information from Sol Genomics Network (Fernandez-Pozo et al., 2015) at www.solgenomics.net and facilitated by the use of Primer3 (Untergasser et al., 2012; Koressaar and Remm, 2007; http://primer3.ut.ee/) and NetPrimer software (PREMIER Biosoft; http://www.premierbiosoft.com/netprimer/).

Microarray Analysis

Potato cv Desiree plants were grown from tubers under controlled conditions with 16 h of light at 18°C and 0% humidity. Epibrassinolide (Sigma) was first solubilized at 20 μg in ethanol before dilution in distilled water. Six-week-old plants were sprayed with a fine mist of 50 μM EBL or a mock control containing distilled water and ethanol only. Leaf tissue was collected 3 and 24 h after EBL/mock treatment and immediately frozen in liquid nitrogen, with three compound leaves harvested for each treatment at each timepoint. Material from four biological replicates was taken forward for microarray analysis. RNA extraction was carried out as above, with sample integrity assessed using a Bioanalyzer (Agilent Technologies). RNA was labeled using the Agilent Two Color Low Input Quick Amp Labeling Kit (v 6.5; as recommended) and, following purification, cRNA hybridized to custom JHI potato 60K microarrays (Agilent pres sure accessions A01176 and A02772) overnight. Arrays were washed and scanned using an Agilent G250 5B scanner, prior to data extraction using Agilent FE software and analysis in GeneSpring (v 7.3 Agilent Technologies; ArrayExpress data accession E-MTAB-3854). Data were normalized using default Lowess settings prior to reimporting into Genespring as individual samples. Filtering was performed to remove those probes with no detectable expression, and statistically significant gene expression between treatments was identified using volcano filtering (t-test P value < 0.05; fold-change > 2x).

Constructs and Cloning

StCHL1 was synthesized with Gateway sites (Eurofins Scientific) and recombined into the entry vector pDONR201 using BP clonase (Invitrogen), followed by recombination into the GFP-tagged vector pB7WG2F (Karimi et al., 2002) using LR clonase (Invitrogen). GFP-AVR2 and pGRAB-AVR2 were
cloned from P. infestans as previously described (Gilroy et al., 2011a), as were 35S-INF1 and GFP-AVR3a (Gilroy et al., 2011b).

**Sequence Analysis and Gene Ontology**

Functional categories were assigned to potato transcripts using Mapman (Thimmapunya et al., 2004). BLAST analysis and sequence acquisition from the Arabidopsis Information Resource (Lamesch et al., 2012) and the Solanum Genome Network (Fernandez-Pozo et al., 2015). Protein domain prediction was facilitated by the Pfam protein families database (Finn et al., 2014). Bayesian (MrBayes) tree was generated using Topali v2.5 on full-length amino acid sequences (Bioinformatics and Statistic).

**SUPPLEMENTAL DATA**

The following supplemental materials are available.

**Supplemental Figure S1.** Transgenic potato lines expressing AVR2.

**Supplemental Figure S2.** Epidermal cell size is increased in 35S:AVR2 expressing transgenic potato lines #29 and #39.

**Supplemental Figure S3.** Protein alignment of CHL1 orthologs.

**Supplemental Figure S4.** SICH1 is closely related to several bHLH DNA binding proteins from Arabidopsis.

**Supplemental Figure S5.** GFP-StCHL1 is stably expressed in N. benthamiana.

**Supplemental Figure S6.** NCCHL1 silencing vector design, plant phenotypes and silencing levels in N. benthamiana.

**Supplemental Table S1.** List of top 50 genes up-regulated in EBL vs water-sprayed potato cv Desiree at 3 h.

**Supplemental Table S2.** List of top 50 genes down-regulated in EBL vs water-sprayed potato cv Desiree at 3 h.

**Supplemental Table S3.** List of top 50 genes up-regulated in EBL vs water-sprayed potato cv Desiree at 24 h.

**Supplemental Table S4.** List of top 50 genes down-regulated in EBL vs water-sprayed potato cv Desiree at 24 h.

**Supplemental Table S5.** Primers used in this study.

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**LITERATURE CITED**


**PiAVR2 Up-Regulates BR Responsive bHLH**


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