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1 **Potato NPH3/RPT2-like protein StNRL1, targeted by a**  
2 ***Phytophthora infestans* RXLR effector, is a susceptibility factor**  
3

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17  
18 **Author Contributions:**

19 E.M.G., H.M., P.C.B., Z.T., J.Z. and P.R.J.B. conceived of and designed the  
20 experiments. L.Y., L.G., P.C.B., H.M., M.A., Q.H., W.Z and S.N. performed the  
21 experiments. L.Y., H.M., M.A., Q.H., S.N., E.M.G. and P.R.J.B. analysed and  
22 interpreted the results. L.Y., H.M. and P.R.J.B. wrote the paper. P.R.J.B. and E.M.  
23 directed the project.

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32 **Abstract**

33 **Plant pathogens deliver effectors to manipulate host processes. We know little**  
34 **about how fungal and oomycete effectors target host proteins to promote**  
35 **susceptibility, yet such knowledge is vital to understand crop disease. We**  
36 **show that either transient expression in *Nicotiana benthamiana*, or stable**  
37 **transgenic expression in potato (*Solanum tuberosum*), of *Phytophthora***  
38 ***infestans* RXLR effector Pi02860 enhances leaf colonization by the pathogen.**  
39 **Expression of Pi02860 also attenuates cell death triggered by the *P. infestans***  
40 **MAMP INF1, indicating that the effector suppresses pattern-triggered immunity**  
41 **(PTI). However, the effector does not attenuate cell death triggered by Cf4/Avr4**  
42 **co-expression, showing that it does not suppress all cell death activated by**  
43 **cell surface receptors. Pi02860 interacts in yeast-2-hybrid assays with potato**  
44 **NPH3/RPT2-like 1 (NRL1), a predicted Cullin-3-associated ubiquitin E3 ligase.**  
45 **Interaction of Pi02860 *in planta* was confirmed by co-immunoprecipitation and**  
46 **bimolecular fluorescence complementation assays. Virus-induced gene**  
47 **silencing (VIGS) of *NRL1* in *N. benthamiana* resulted in reduced *P. infestans***  
48 **colonization and accelerated INF1-mediated cell death, indicating that this host**  
49 **protein acts as a negative regulator of immunity. Moreover, whereas *NRL1***  
50 **VIGS had no effect on the ability of *P. infestans* effector AVR3a to suppress**  
51 **INF1-mediated cell death, such suppression by Pi02860 was significantly**  
52 **attenuated, indicating that this activity of Pi02860 is mediated by NRL1.**  
53 **Transient overexpression of NRL1 resulted in suppression of INF1-mediated**  
54 **cell death and enhanced *P. infestans* leaf colonization, demonstrating that**  
55 **NRL1 acts as a susceptibility factor to promote late blight disease.**

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## 60 Introduction

61 Plant immunity is triggered by detection of conserved microbial molecules, microbe-  
62 or pathogen-associated molecular patterns (M/PAMPs), leading to pattern-triggered  
63 immunity (PTI), and by the detection of effectors, leading to effector-triggered  
64 immunity (ETI). Central to the successful colonisation of plants by phytopathogens is  
65 the delivery of effector proteins to suppress host immunity. Secreted effectors may  
66 act outside (apoplastic effectors) or be delivered inside (intracellular or cytoplasmic  
67 effectors) host cells to attenuate PTI or ETI (Jones and Dangl 2006). A broad range  
68 of host targets and activities have been elucidated for many bacterial type 3  
69 secretion system effectors (Block and Alfano 2011; Deslandes and Rivas 2012; Dou  
70 and Zhou 2012). In contrast, less is understood about the effectors from filamentous  
71 phytopathogens: the fungi and oomycetes.

72 The oomycete *Phytophthora infestans* causes the devastating late blight disease of  
73 potato and tomato (Kamoun et al. 2014). Amongst the classes of candidate virulence  
74 determinants that have been identified are the RXLR effectors (Birch et al. 2006),  
75 which are delivered inside living plant cells (Whisson et al. 2007). Following the  
76 identification of the ubiquitin E3 ligase CMPG1 as a target of AVR3a (Bos et al.  
77 2010), the targets and/or virulence activities of a small number of other *P. infestans*  
78 RXLRs have been revealed. AvrBlb2 prevents the secretion of a defence protease  
79 (Bozkurt et al. 2011), whilst AVR2 interaction with the putative phosphatase BSL1,  
80 involved in the brassinosteroid signal transduction pathway, facilitates recognition of  
81 the effector by the resistance protein R2 (Saunders et al. 2012). Effector Pi03192  
82 interacts with NAC transcription factors, preventing their re-localisation from the  
83 endoplasmic reticulum to the host nucleus (McLellan et al. 2013). PexRD2 targets  
84 the host MAP3K $\epsilon$ , inhibiting signal transduction following perception of Cf-Avr4 from  
85 *Cladosporium fulvum* by tomato resistance Cf4 (King et al. 2014), whilst a number of  
86 effectors act redundantly to suppress flg22-mediated MAPK activation and early  
87 transcriptional changes (Zheng et al. 2014), implicating this signal transduction  
88 pathway also in response to unknown oomycete MAMPs. A K-homology class RNA  
89 binding protein, StKRBP1, which associates with RXLR effector Pi04089, provides  
90 the first evidence that *P. infestans* effectors manipulate host susceptibility factors to  
91 promote late blight disease (Wang et al. 2015). Pi04089 increases the abundance of

92 StKRBP1, a phenomenon which also occurs during the first 24 hours of *P. infestans*  
93 infection. Overexpression of this RNA binding protein enhances leaf colonisation by  
94 the pathogen (Wang et al. 2015). More recently, an RXLR effector from *P. infestans*  
95 has been shown to target host PP1c isoforms. Rather than inhibiting these  
96 phosphatases the effector forms unique holoenzymes with them to presumably  
97 dephosphorylate key substrates in the plant nucleus, leading to enhanced  
98 susceptibility (Boevink et al 2016). The PP1c isoforms can thus also be regarded as  
99 susceptibility factors.

100 One of the key *P. infestans* MAMPs detected by solanaceous hosts is INF1, which  
101 elicits BAK1-dependent cell death in the model host plant *Nicotiana benthamiana*  
102 (Heese et al. 2007) and a range of *Solanum* species (Vleeshouwers et al. 2006).  
103 Recently, a receptor that detects INF1 and other elicitors from a broad range of  
104 oomycetes, termed ELR, has been cloned from *Solanum microdontum* (Du et al.  
105 2015). Overexpression of ELR in the cultivated potato enhances disease resistance  
106 to *P. infestans* (Du et al. 2015). INF1-mediated cell death can be suppressed by  
107 AVR3a, either by inhibition or modification of CMPG1 activity (Bos et al. 2010; Gilroy  
108 et al. 2011), and can be partially suppressed by RXLR effector Pi18215/SFI7, which  
109 also inhibits flg22-mediated MAPK activation (Zheng et al. 2014).

110 In addition to CMPG1, another plant U-box (PUB) ubiquitin E3 ligase, PUB17, has  
111 been shown to positively regulate immunity (Yang et al 2006). PUB17 functions in  
112 the host nucleus to mediate both PTI, following perception of bacterial PAMP flg22,  
113 and cell death triggered by co-expression of Cf4/Avr4. However, it is not involved in  
114 INF1-triggered cell death (He et al 2015). In contrast to CMPG1 and PUB17, a  
115 number of PUB E3 ligases have been shown to negatively regulate plant immunity  
116 (Duplan and Rivas, 2014). PUB12 and PUB13 work in concert to attenuate PTI by  
117 ubiquitinating the flg22 receptor FLS2, facilitating its degradation (Lu et al 2011).  
118 PUB22, PUB23, and PUB24 also act to suppress immunity. In addition, NPR3 and  
119 NPR4 contain Broad-Complex, Tramtrack and Bric-a-brac (BTB) domains that  
120 facilitate interaction with cullin-3 E3 ligase. NPR3 and NPR4 negatively regulate  
121 salicylic acid-mediated defences (Fu et al 2012). Ubiquitination is thus a post-  
122 translational modification implicated in both positive and negative regulation of  
123 immunity.

124 Here we show that either transient expression in model host *Nicotiana benthamiana*,  
125 or stable transformation in potato, of putative RXLR effector PITG\_02860 (Pi02860)  
126 supports enhanced leaf colonisation by *P. infestans*. Expression of the effector in *N.*  
127 *benthamiana* also suppresses INF1-mediated cell death, indicating that Pi02860  
128 contributes to PTI suppression. Pi02860 localises to the cytoplasm in *N.*  
129 *benthamiana*. Pi02860 interacts with an NPH3/RPT2-like (NRL) protein, StNRL1, in  
130 yeast-2-hybrid assays and *in planta*. Virus induced gene silencing (VIGS) of *NbNRL1*  
131 attenuates *P. infestans* colonisation and accelerates INF1-mediated cell death.  
132 Moreover, VIGS of *NbNRL1* prevents the ability of Pi02860 to suppress INF1-  
133 mediated cell death, whereas such suppression by AVR3a is unaltered, indicating  
134 that PTI suppression by Pi02860 is mediated by NRL1. In contrast, overexpression  
135 of StNRL1 alone enhances colonisation and suppresses INF1-mediated cell death,  
136 indicating that StNRL1 is a negative regulator of PTI and can thus be regarded as a  
137 susceptibility factor.

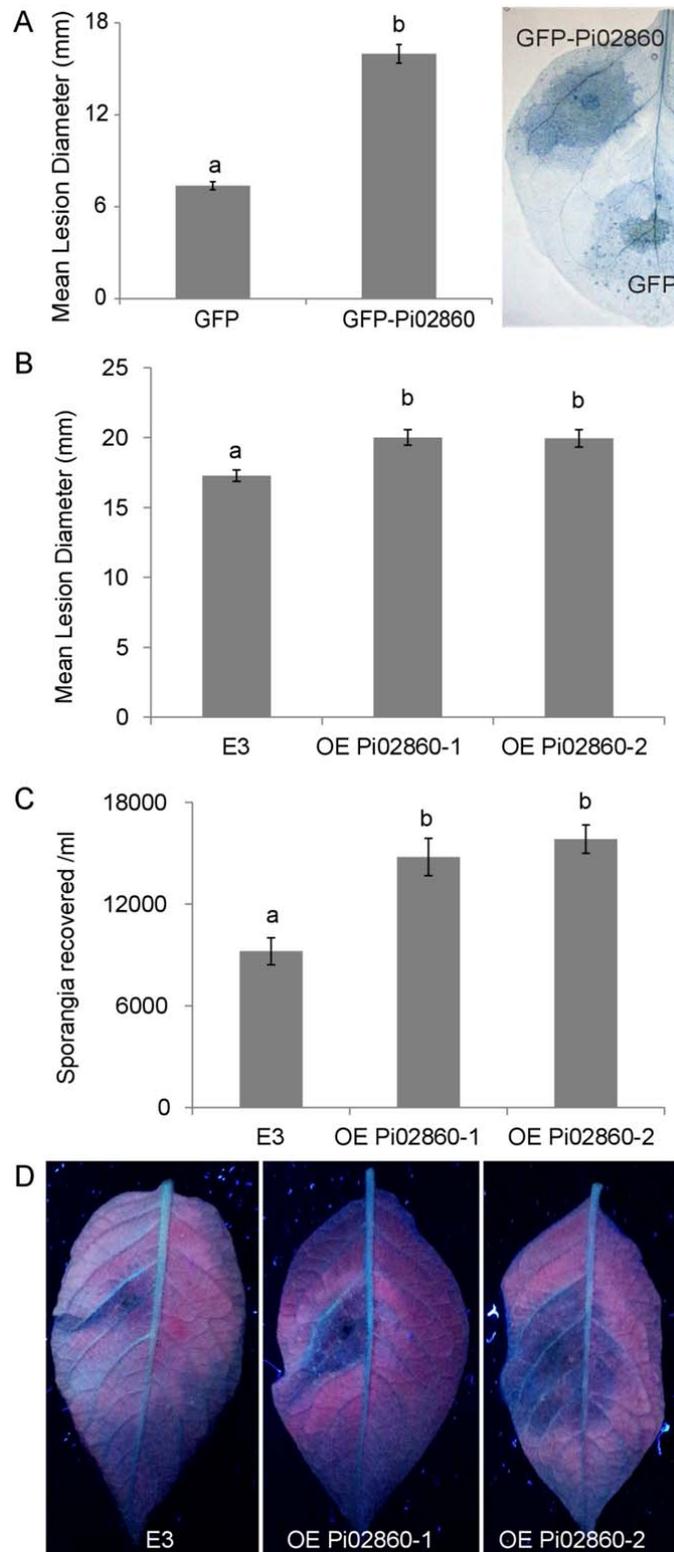
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## 139 Results and Discussion

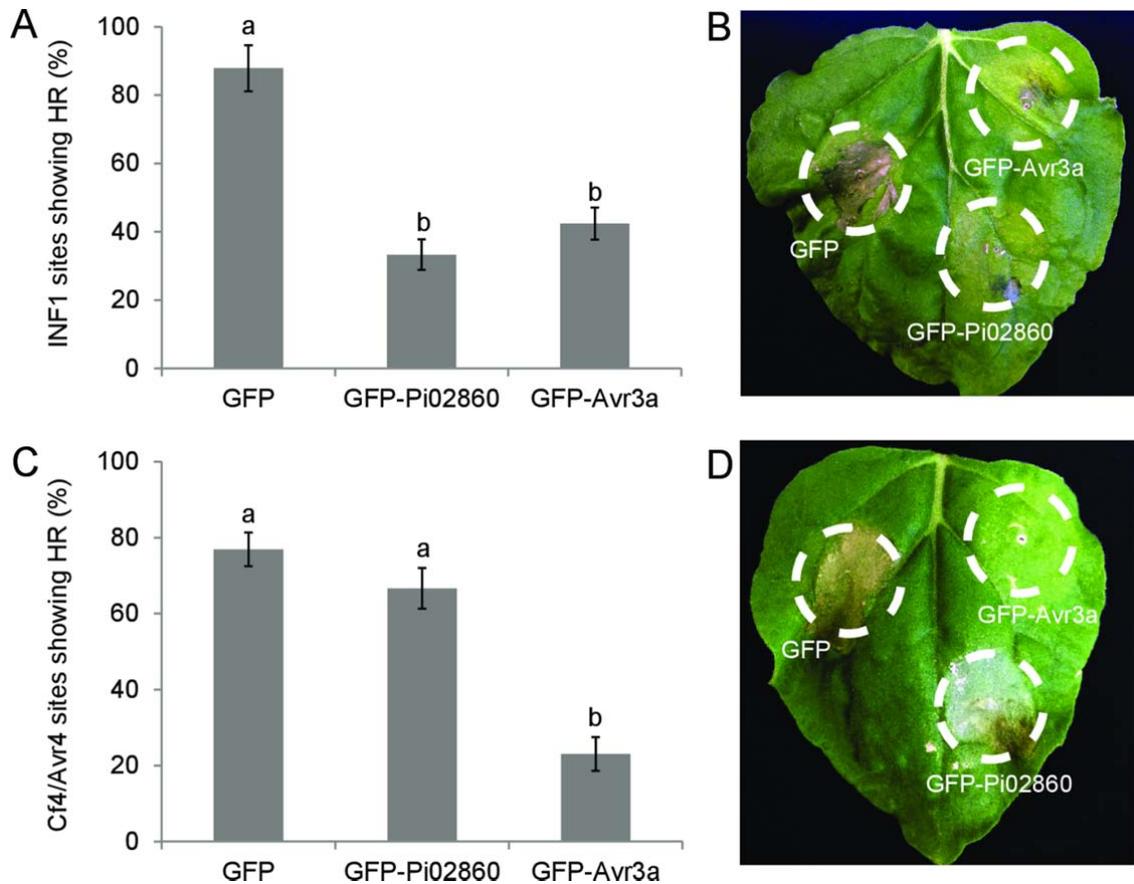
### 140 Pi02860 promotes *P. infestans* virulence and suppresses PTI

141 The effector Pi02860 (PITG\_02860) is annotated in the *P. infestans* genome as a  
142 secreted RXLR type effector protein (Haas et al. 2009). Consistent with other RXLR  
143 effectors, *Pi02860* is specifically up-regulated at two and three days post-infection  
144 (dpi) of potato plants challenged with distinct *P. infestans* genotypes (Haas et al.  
145 2009; Cooke et al. 2012). As these time-points correspond to the biotrophic phase of  
146 *Phytophthora* infection (Avrova et al. 2008) this effector was cloned and tested for its  
147 ability to influence *P. infestans* colonisation. A construct with GFP fused to the N-  
148 terminus of Pi02860 in place of the signal peptide was cloned and transiently  
149 expressed in the model solanaceous *P. infestans* host plant *N. benthamiana* using  
150 *Agrobacterium*-mediated expression followed by *P. infestans* challenge, as  
151 performed for other RXLR effectors (McLellan et al. 2013; Zheng et al. 2014; King et  
152 al. 2014). At 6 dpi significantly larger lesions (ANOVA,  $p < 0.001$ ) were observed in  
153 areas expressing GFP-Pi02860 compared to the expression of free GFP (Fig. 1A)  
154 thus suggesting that Pi02860 confers a benefit to the pathogen consistent with  
155 effector activity. To explore this phenomenon further in the host crop plant,  
156 transgenic potato lines were made for stable expression of Pi02860, minus signal  
157 peptide-encoding sequences (Supplemental Fig. S1). These plants were  
158 subsequently challenged with *P. infestans* and were also found to support  
159 significantly larger lesions (ANOVA,  $p < 0.002$ ) (Fig. 1B; 1D), and significantly  
160 enhanced sporulation (ANOVA,  $p < 0.001$ ) of the pathogen (Fig. 1C). The  
161 enhancement of *P. infestans* leaf colonisation promoted by Pi02860 expression  
162 inside host cells is similar to other recently described RXLR effectors (McLellan et al.  
163 2013; Zheng et al. 2014; King et al. 2014; Wang et al. 2015; Boevink et al 2016) and  
164 consistent with it modifying the host to promote susceptibility.

165 As some RXLR effectors have been demonstrated to interfere with distinct defence  
166 signalling pathways *in planta* (e.g. Bos et al. 2010; King et al. 2014) GFP-Pi02860  
167 was tested to determine if it attenuated cell death signalling activated by two  
168 characterised pathways. The *Phytophthora* PAMP INF1 triggers a hypersensitive  
169 response (HR) in some solanaceous hosts, including *N. benthamiana*, and this HR  
170 can be blocked by co-expressing RXLR effector AVR3a (Bos et al. 2010). In

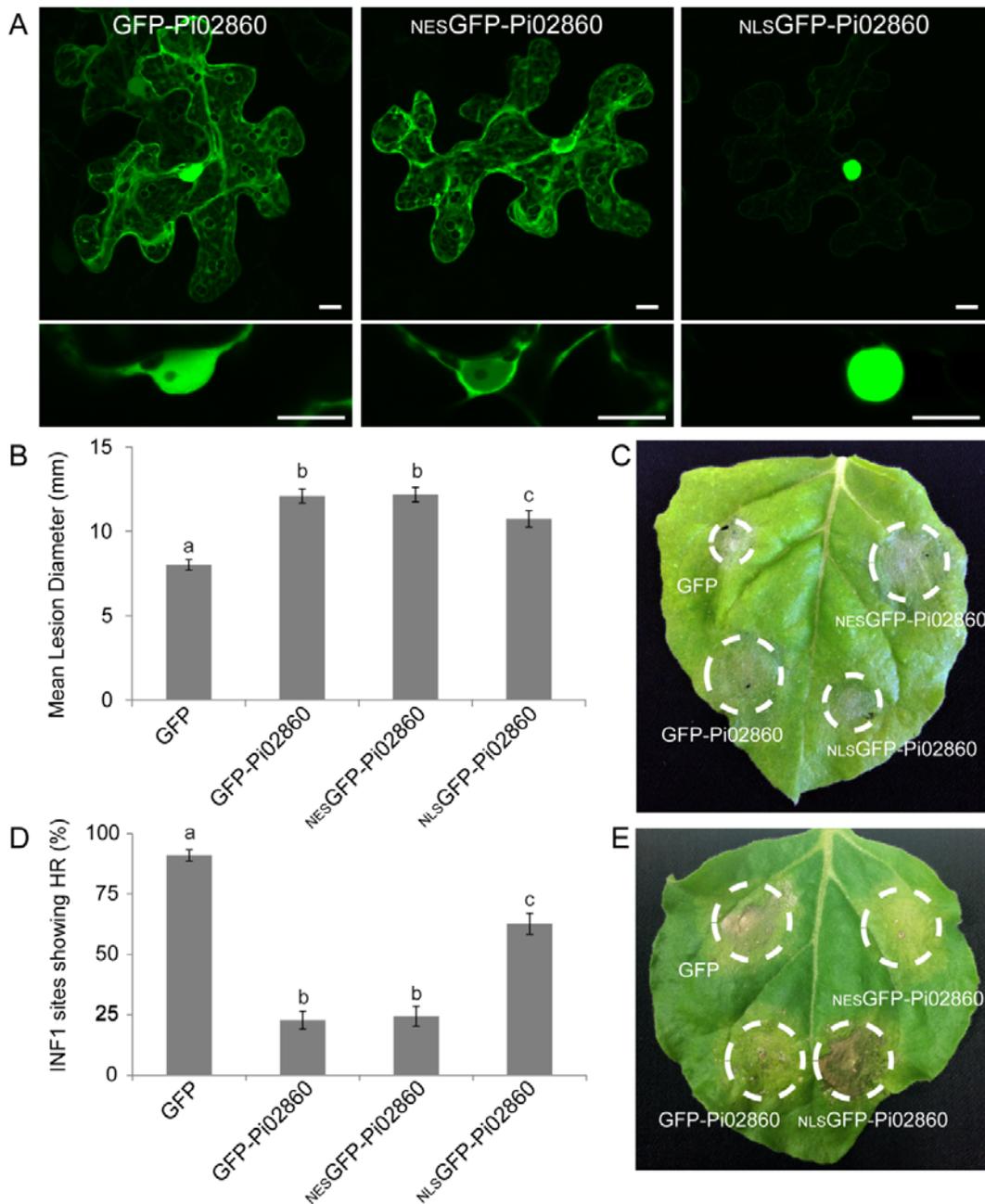


171 addition, effectors AVR3a and PexRD2 are both able to suppress the HR triggered  
 172 by co-expression of the *Cladosporium fulvum* effector Avr4 and its cognate  
 173 resistance protein Cf4 by different mechanisms (Bos et al. 2010; King et al. 2014).  
 174 Expression of GFP-Pi02860 was found to significantly attenuate INF1-mediated HR



175 (ANOVA,  $p < 0.001$ ) to a similar level as the GFP-AVR3a control (Fig. 2A; 2B). In  
 176 contrast to GFP-AVR3a, it had no significant effect on Cf4-CfAvr4 HR ( $p > 0.1$ ) (Fig.  
 177 2C; 2D). This suggests that the function of Pi02860 may be to suppress a specific  
 178 signalling pathway(s) which is triggered on perception of *P. infestans* PAMPs, such  
 179 as INF1, and does not extend to all cell death pathways triggered by activation of cell  
 180 surface receptors.

181 To further investigate the phenotypes associated with Pi02860 overexpression the  
 182 subcellular localisation of this protein was examined in *N. benthamiana* using  
 183 confocal microscopy. GFP-Pi02860 was found to localise throughout the plant  
 184 cytoplasm and nucleoplasm (Fig. 3A). To perturb the observed localisation of the  
 185 effector two additional fusion constructs were generated, to which either a nuclear  
 186 export signal (NES) or nuclear localisation signal (NLS) were added to the N-  
 187 terminally fused GFP, as described previously (Wang et al. 2015). Both produced  
 188 intact fusion proteins when expressed *in planta* (Supplemental Fig. S2). On  
 189 examination with confocal microscopy,  $_{NES}$ GFP-Pi02860 was greatly reduced in the  
 190 nucleoplasm but still accumulated in the cytoplasm, while  $_{NLS}$ GFP-Pi02860

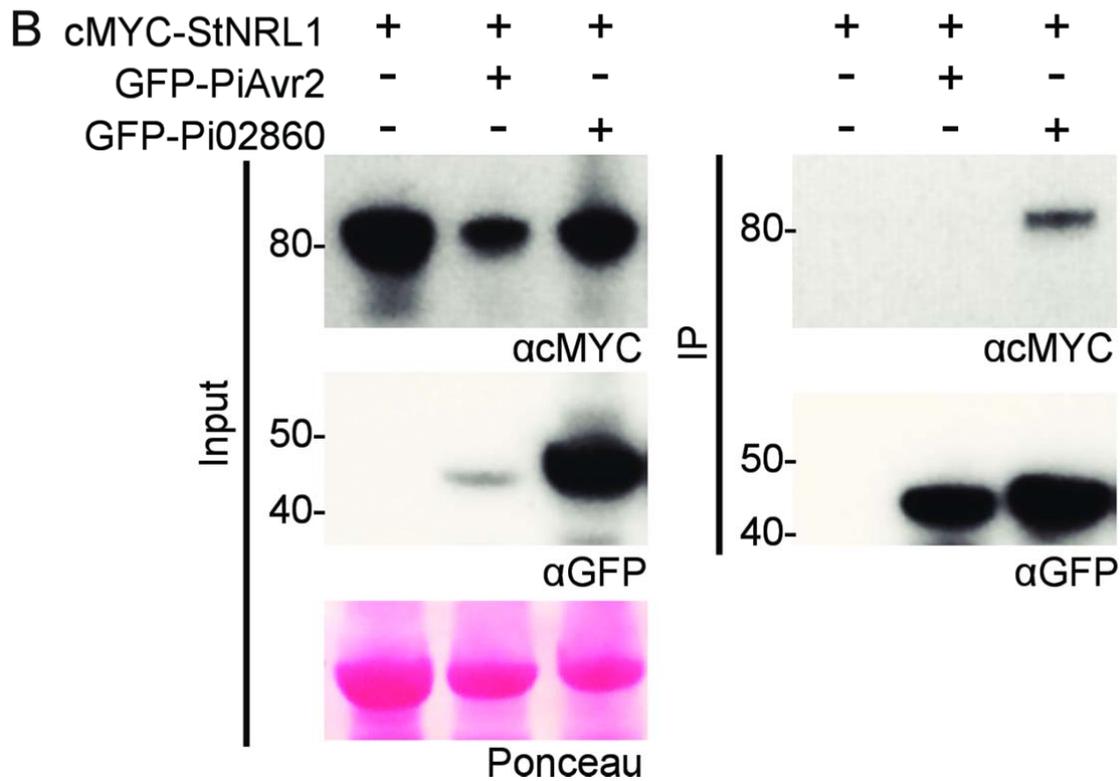
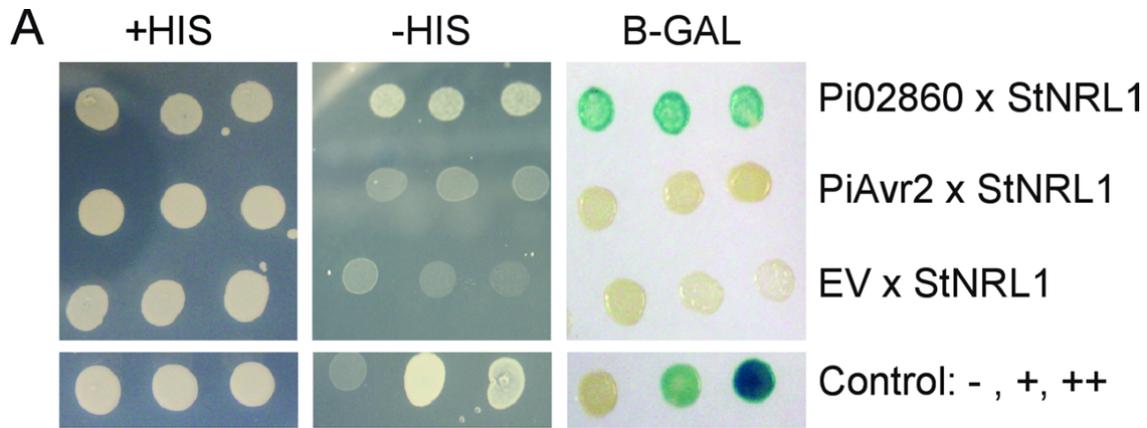


191 accumulated strongly in the nucleus and was largely reduced in the cytoplasm (Fig.  
 192 3A). The effects of these constructs were tested in the *P. infestans* virulence assay,  
 193 using GFP-Pi02860 and GFP as positive and negative controls, respectively.  
 194 Interestingly, expression of <sub>NES</sub>GFP-Pi02860 was found to enhance leaf colonisation  
 195 to the same level as GFP-Pi02860, while lesion sizes following expression of  
 196 <sub>NLS</sub>GFP-Pi02860 were significantly reduced compared to GFP-Pi02860 but were still  
 197 significantly larger than free GFP (Fig. 3B; 3C). A similar pattern was observed when  
 198 testing these GFP constructs for their ability to suppress INF1-mediated HR. Again

199  $_{NES}GFP$ -Pi02860 was found to suppress INF1 HR to similar levels as GFP-Pi02860.  
200 In contrast,  $_{NLS}GFP$ -Pi02860 was significantly less able to suppress INF1-mediated  
201 HR compared to GFP-Pi02860, but the HR was nevertheless more significantly  
202 suppressed compared to that observed with free GFP expression (Fig. 3D; 3E).  
203 Whilst the NES fusion did not totally exclude GFP-Pi02860 from the nucleus, and the  
204 NLS fusion still retained background levels of cytoplasmic fluorescence, these  
205 results may nevertheless indicate that the cytoplasmic localisation of Pi02860 is  
206 more important for its contribution to virulence than the observed nucleoplasmic  
207 localisation.

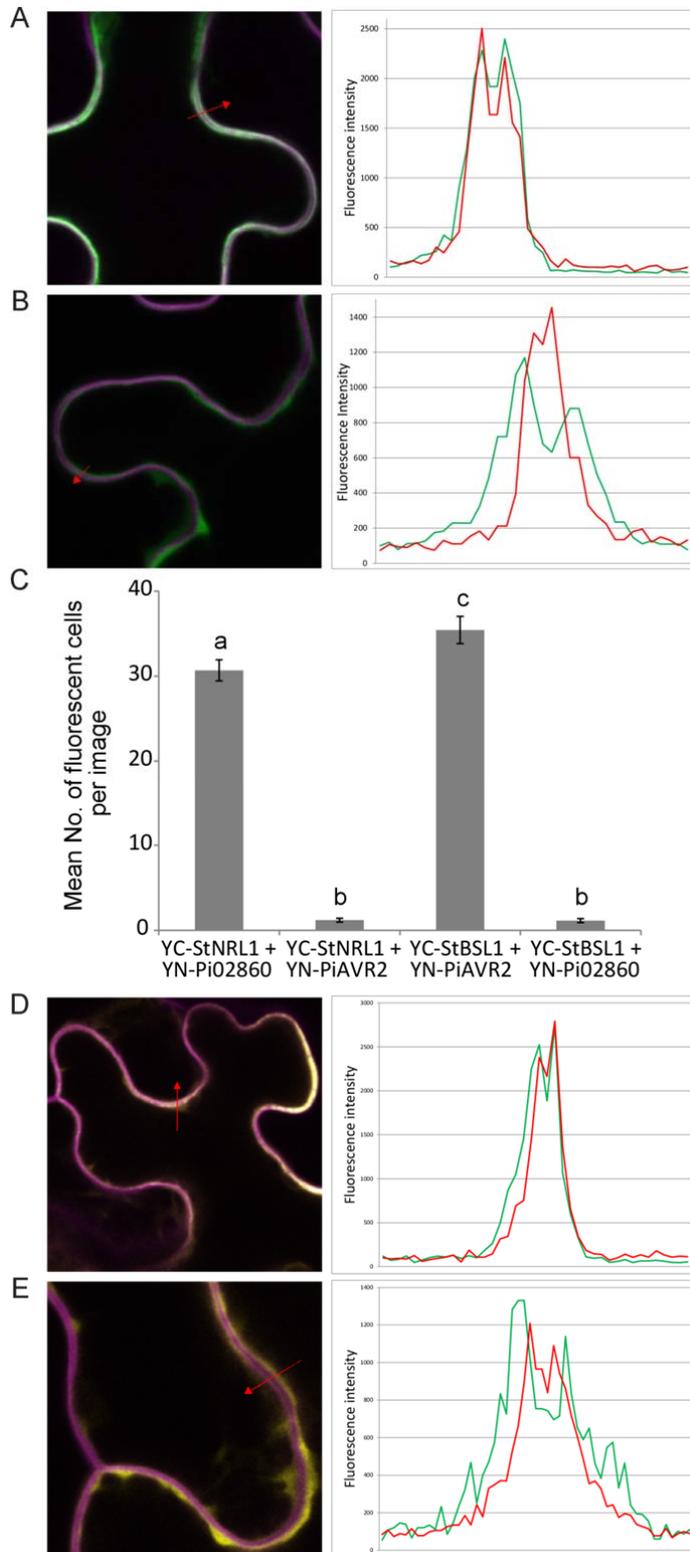
### 208 **Pi02860 interacts with the BTB/POZ domain protein StNRL1**

209 To further explore the mechanism of Pi02860 action in plants, a yeast-2-hybrid (Y2H)  
210 library made from cDNA of potatoes infected with *P. infestans* (Bos et al 2010) was  
211 screened with a GAL4 DNA binding domain-Pi02860 fusion ('bait') construct to a  
212 depth of  $0.44 \times 10^6$  yeast co-transformants. Five yeast colonies recovered from  
213 selection plates that contained GAL4 activation domain ('prey') fusions, yielded  
214 sequences corresponding to a potato Broad-Complex, Tramtrack and Bric-a-brac  
215 (BTB/POZ) domain protein belonging to the Non-phototrophic hypocotyl 3/ Root  
216 phototropism 2 (NPH3/RPT2)-Like family, hereafter referred to as StNRL1. In  
217 Arabidopsis NPH3 and RPT2 interact with phototropins, mediating blue light  
218 signalling, and are thought to be a core component of a cullin-3 (CUL3)-based  
219 ubiquitin-protein ligase (E3) enzyme complex (Liscum et al. 2014). Supplemental  
220 Fig. S3 shows an amino acid alignment of potato StNRL1 with its *N. benthamiana*  
221 equivalents NbNRL1a and NbNRLb, the Arabidopsis protein At5g67385 (AtNRL) that  
222 is a candidate orthologue (reciprocal best BLAST hit), and the characterised  
223 At5g64330 (AtNPH3) and At2g30520 (AtRPT2), indicating the conserved domains  
224 across these proteins. To confirm this interaction, a full length StNRL1 prey construct  
225 was tested pairwise with bait constructs for Pi02860, a non-interacting RXLR control,  
226 PiAVR2, which has been shown previously to associate with the putative  
227 phosphatase BSL1 (Saunders et al. 2012), and the empty bait vector (EV). While all  
228 transformants grew on the control plates (+ HIS) only Yeasts containing both  
229 Pi02860 and StNRL1 were able to grow on the selection (-HIS) plates and activate  
230 the  $\beta$ -galactosidase (B-GAL) reporter (Fig. 4A).



231 To confirm this interaction also occurs *in planta* a co-immunoprecipitation (Co-IP)  
 232 assay was performed by expressing cMyc-tagged StNRL1 (cMyc-StNRL1) alone or  
 233 with GFP-Pi02860 or GFP-PiAVR2 and pulling down with GFP-TRAP\_M beads. Fig.  
 234 4B shows that, while all proteins were present in the relevant input samples, cMYC-  
 235 StNRL1 was only immunoprecipitated in the presence of GFP-Pi02860 and not alone  
 236 or with the GFP-PiAVR2 control.

237 To examine StNRL1 in more detail GFP was fused to its N-terminus to form GFP-  
 238 StNRL1 and was localised in *N. benthamiana* using confocal microscopy. The GFP-  
 239 StNRL1 fusion localised partially in the cytoplasm, but showed significant



240 accumulation at the plasma membrane (PM), when compared to a free GFP control  
 241 (Supplemental Fig. S4). Co-expression of GFP-StNRL1 with an mOrange-LTi PM  
 242 marker indicated significant co-localisation, which was not observed with free GFP

243 (Fig. 5A, 5B; Supplemental Fig. S4). This indicates that, whilst GFP-NRL1 is  
244 observed in the cytoplasm, it also strongly associates with the PM.

245 A bimolecular fluorescence complementation assay (commonly referred to as Split  
246 YFP) was then undertaken to establish the site of the interaction of StNRL1 and  
247 Pi02860 proteins *in planta*. The C-terminus fragment of YFP (YC) was fused to  
248 StNRL1 while the N-terminus (YN) was fused to Pi02860 to give YC-StNRL1 and  
249 YN-Pi02860, respectively. As the controls used in split YFP studies are important to  
250 rule out false positive interactions (Boevink et al. 2014) we used YC-StBSL1 and YN-  
251 PiAVR2 as controls for a plant target and effector interacting pair which also localise  
252 to the plant cytoplasm and PM (Saunders et al. 2012). Co-expression of either YC-  
253 StNRL1 with YN-Pi02860 or YC-StBSL1 with YN-PiAVR2 yielded fluorescence  
254 visualised by confocal microscopy, whereas there was no appreciable fluorescence  
255 when the YN-Pi02860 was co-expressed with YC-BSL1, or when YN-AVR2 was co-  
256 expressed with YC-NRL1 (Supplemental Fig. S4). This was quantified by counting  
257 the number of fluorescent cells in the field of view in >50 low magnification images  
258 each to show that YC-StNRL1 with YN-Pi02860 or YC-StBSL1 with YN-PiAVR2  
259 gave significantly higher fluorescence (ANOVA,  $p < 0.001$ ) than YC-StNRL1 with YN-  
260 PiAVR2 or YC-StBSL1 with YN-Pi02860 (Fig. 5C).

261 The YC-StNRL1 and YN-Pi02860 constructs were co-expressed in *N. benthamiana*  
262 with the mOrange-LTi PM marker and YFP fluorescence was observed in the  
263 cytoplasm, but with significant accumulation at the PM using confocal microscopy,  
264 compared to free YFP control (Fig. 5D, 5E; Supplemental Fig. S4). The presence of  
265 each of the intact fusion constructs was confirmed by immunoblotting to rule out  
266 changes in fluorescence levels being caused by construct instability (Supplemental  
267 Fig. S5).

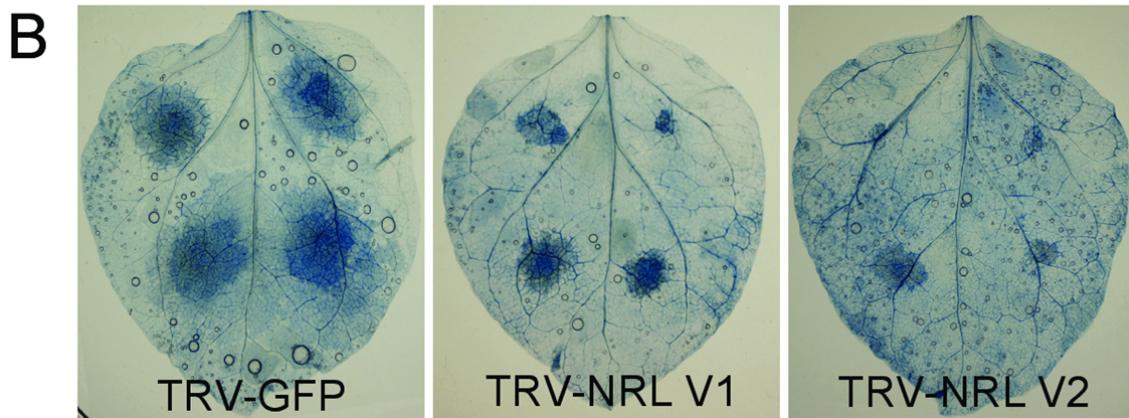
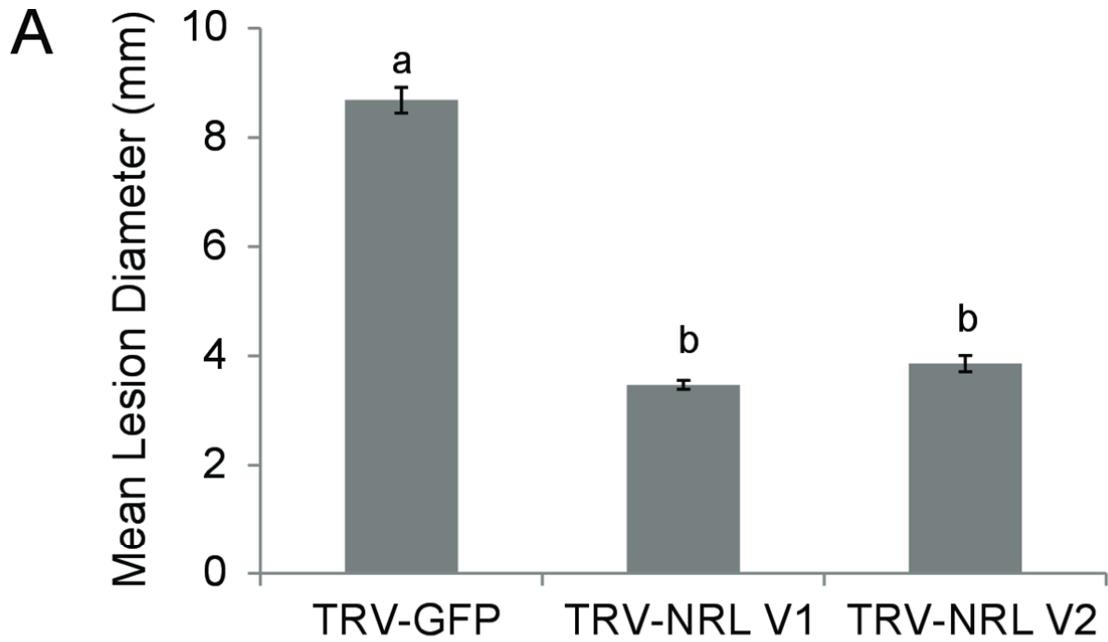
268 The localisation of StNRL1 at the host PM is in agreement with studies of NRL family  
269 members in Arabidopsis, all of which have been shown to interact at the PM, as part  
270 of a cullin-3 (CUL3) RING ubiquitin ligase (CRL) complex, with phototropins, which  
271 are involved in blue light signalling (Liscum et al. 2014).

272 **StNRL1 silencing retards *P. infestans* colonisation and prevents Pi02860**  
273 **suppression of INF1-mediated cell death**

274 To examine a possible role for StNRL1 in plant defence against *P. infestans*, virus  
275 induced gene silencing (VIGS) was used to knock down the expression of the  
276 equivalent *NRL1* genes in *N. benthamiana*. *N. benthamiana* is an allotetraploid and  
277 thus gene searches in the genome usually reveal two matching copies where the two  
278 homeologous genes have not been collapsed during assembly (Bombarely et al  
279 2012). Consistent with that, two sequences, designated *NbNRL1a* and *NbNRL1b*,  
280 encoding proteins with 95 % amino acid identity to each other, were identified in the  
281 *N. benthamiana* genome. The predicted *NbNRL1a* and *NbNRL1b* proteins are each  
282 84 % identical to StNRL1 (Supplemental Fig. S3). Consequently, two independent  
283 VIGS constructs, TRV-NRL V1 and TRV-NRL V2, were designed to silence both  
284 homeologous copies simultaneously. Supplemental Fig. S6A shows that transcript  
285 accumulation of both *NbNRL1a* and *NbNRL1b* are reduced by 60-85% in plants  
286 expressing either TRV-NRL construct, compared to plants expressing the TRV-GFP  
287 control. Representative images of plants expressing each TRV-NRL VIGS construct  
288 show that these plants exhibit a developmental phenotype, being stunted in growth  
289 compared to the TRV-GFP control (Supplemental Fig. S6B).

290 Following infection of the VIGS plants with *P. infestans* it was observed that silencing  
291 of *NbNRL1a* and *NbNRL1b* led to a reduction in the ability of the pathogen to  
292 colonise these plants, with significantly smaller lesions (ANOVA,  $p < 0.001$ )  
293 developing on TRV-NRL plants compared to the TRV-GFP controls (Fig. 6A & 6B).  
294 This suggests that *P. infestans* requires the presence of *NRL1* to establish normal  
295 infections, and would not support a model in which effector Pi02860 inhibits or  
296 inactivates *NRL1*.

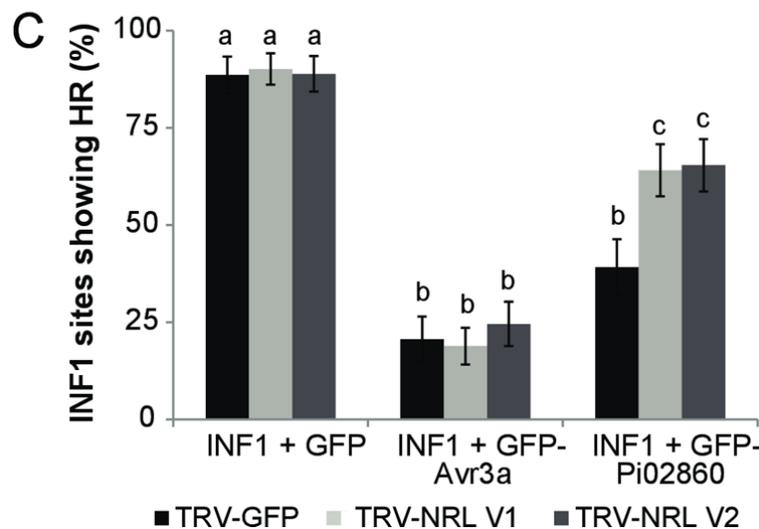
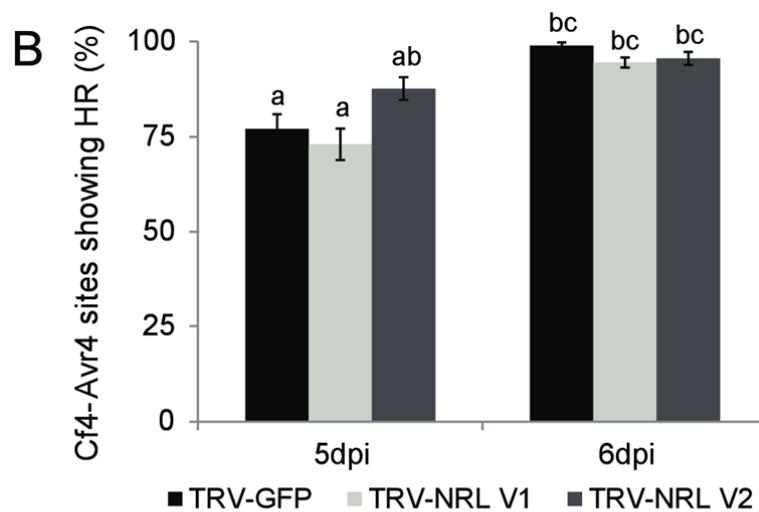
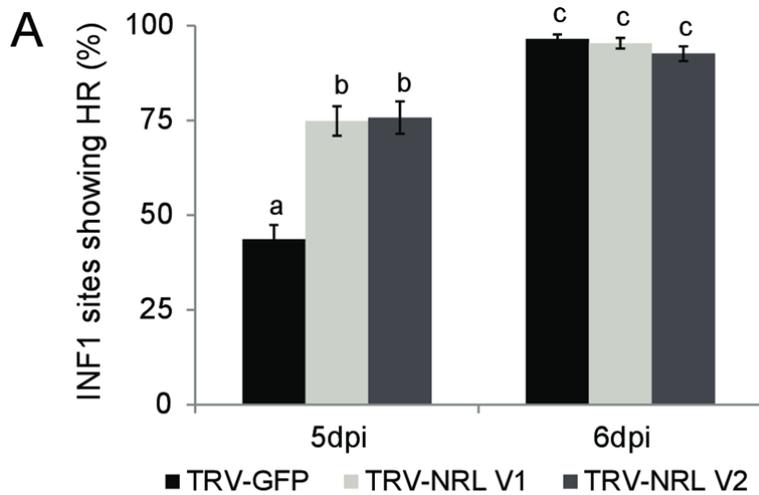
297 As Pi02860 was also observed to suppress INF1-mediated cell death, constructs  
298 expressing INF1 were agro-infiltrated into plants expressing each of the VIGS  
299 constructs and scored at 5 and 6 days post-inoculation (dpi). After 5 dpi a significant  
300 increase (ANOVA,  $p < 0.001$ ) in INF1 HR was observed on TRV-NRL VIGS plants  
301 compared to TRV-GFP (Fig. 7A). This difference was not apparent by 6 dpi  
302 indicating that the HR is accelerated by *NbNRL1* silencing. The same assay was  
303 carried out to examine the Cf4-Avr4 HR, which was not effected by Pi02860  
304 expression. As anticipated, there were no significant differences in Cf4-Avr4 HR in  
305 TRV-NRL plants compared to TRV-GFP at either 5 or 6 dpi (Fig. 7B). These results  
306 suggest that *NRL1* acts as a negative regulator of INF1-mediated cell death.



307 To investigate whether suppression of INF1-mediated cell death by Pi02860 is  
 308 dependent on the presence of NRL1, either GFP-Pi02860 or, as a control, GFP-  
 309 AVR3a, was co-expressed with INF1 in leaves expressing either the TRV-NRL VIGS  
 310 constructs or TRV-GFP. Whereas GFP-AVR3a suppressed INF1-mediated cell

311 death to similar amounts on all plants, suppression of INF1-mediated cell death by  
312 GFP-Pi02860 was retained on TRV-GFP plants, but was significantly reduced on  
313 plants in which *NbNRL1a* and *NbNRL1b* were silenced (Fig. 7C). Some ability to  
314 suppress INF1-mediated cell death was retained. However, this is likely due to the

315 fact that VIGS is notoriously 'patchy', with some leaf areas more efficiently silenced  
316 than others, and that silencing knocked down the transcript levels of *NbNRL1a* and  
317 *NbNRL1b* by 60-85 % (Supplemental Fig. S6), suggesting that some NRL1 protein is  
318 likely present. Nevertheless, the significant reduction in INF1 cell death suppression



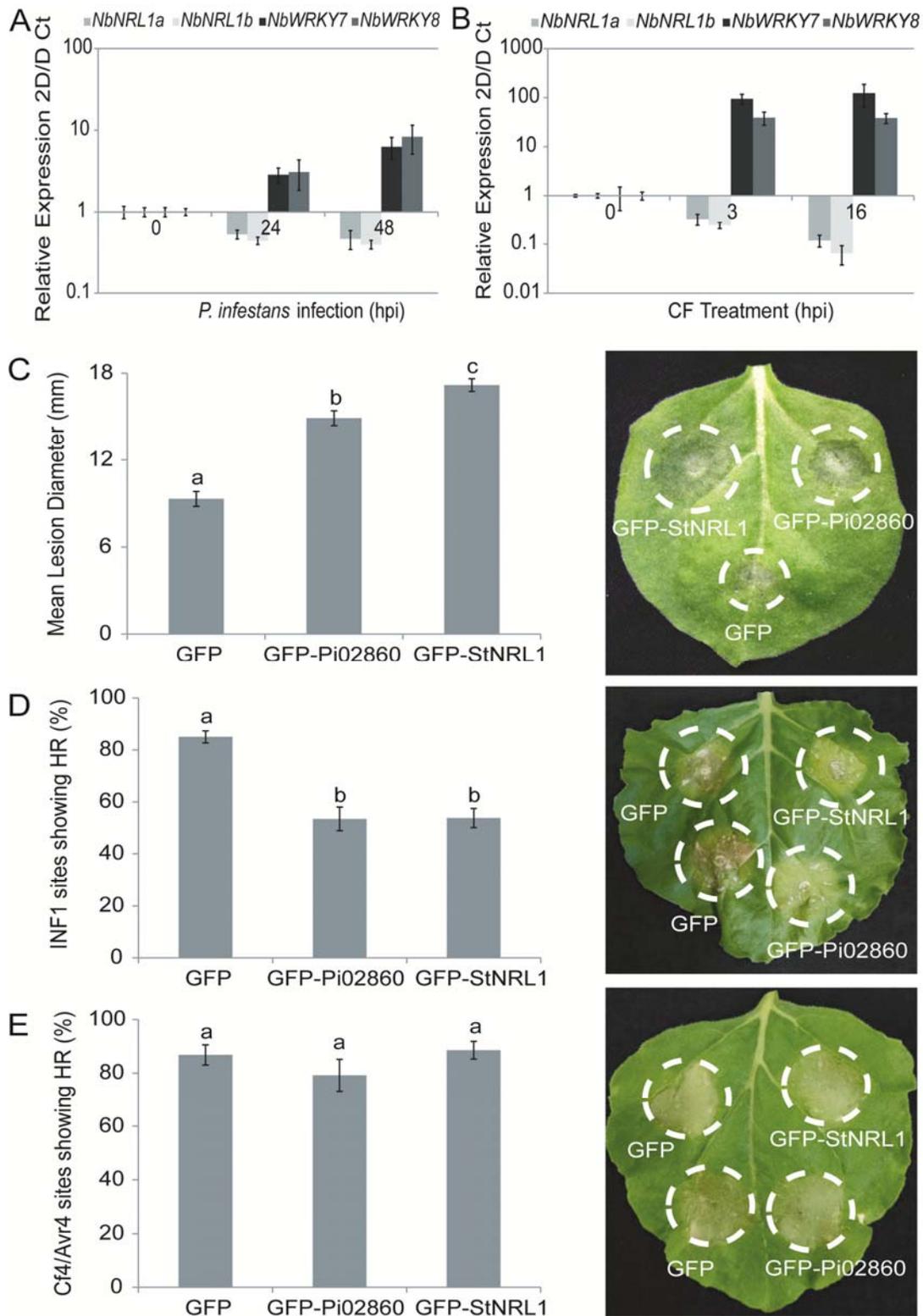
319 by GFP-Pi02860 provides a direct genetic link to indicate that this effector activity is  
 320 dependent on the presence of NRL1. Taken together, the results indicate that NRL1  
 321 is a negative regulator of immunity and is thus unlikely to be inhibited by Pi02860.

322 **StNRL1 overexpression suppresses INF1-mediated cell death and enhances *P.***  
323 ***infestans* colonisation**

324 Silencing of *NbNRL1a* and *NbNRL1b* by VIGS in *N. benthamiana* led to accelerated  
325 INF1-triggered cell death and reduced *P. infestans* colonisation, suggesting that  
326 NRL1 acts as a negative regulator of immunity. We thus investigated the expression  
327 of *NbNRL1a* and *NbNRL1b* during the first 48 hours of *P. infestans* colonisation of *N.*  
328 *benthamiana*, which can be regarded as the biotrophic phase of infection (Avrova et  
329 al 2008), and at times of 3 and 16 hours post-treatment with *P. infestans* culture  
330 filtrate (CF), which can be regarded as a cocktail of *Phytophthora* PAMPs (McLellan  
331 et al 2013). In contrast to two PTI marker genes, *NbWRKY7* and *NbWRKY8*, which  
332 were, similar to previous observations (McLellan et al 2013), weakly up-regulated  
333 during infection and strongly up-regulated by CF treatment, transcript accumulation  
334 of both *NbNRL1a* and *NbNRL1b* decreased weakly during infection and strongly with  
335 CF treatment (Fig 8A; 8B), indicating they are potentially down-regulated during  
336 immune responses. This is consistent with NRL1 acting as a negative regulator of  
337 immunity. To further investigate this we studied the effects of NRL1 overexpression.

338 Transient expression in *N. benthamiana* of either GFP-StNRL1 or GFP-Pi02860,  
339 followed by pathogen challenge was found to result in significantly larger *P. infestans*  
340 lesions ( $p < 0.001$ ) compared to free GFP expression, with GFP-StNRL1  
341 overexpression having a larger infection-enhancing effect on *P. infestans* growth  
342 than GFP-Pi02860 (Fig. 8C). Moreover, either GFP-StNRL1 or GFP-Pi02860  
343 expression independently suppressed INF1-mediated HR to a similarly significant  
344 level ( $p < 0.001$ ) compared to free GFP expression (Fig. 8D). In contrast, expression  
345 of either GFP-StNRL1 or GFP-Pi02860 had no significant effect on Cf4-Avr4-  
346 mediated HR (Fig. 8E).

347 Recently, the *P. infestans* effector Pi04089 has been shown to interact with a KH  
348 RNA binding protein, KRBP1, which is a susceptibility factor. KRBP1 protein turnover  
349 is reduced in the presence of Pi04089, suggesting that the effector enhances its  
350 stability (Wang et al 2015). We thus investigated whether such a phenomenon  
351 occurred with StNRL1 in the presence of Pi02860. However, in three independent  
352 replicates, GFP-StNRL1 protein stability was not enhanced by co-expression with  
353 cMYC-Pi02860, compared to a cMYC empty vector control (Supplemental Fig. S7).



354 This work shows that the *P. infestans* RXLR effector Pi02860, when expressed *in*  
 355 *planta*, enhances pathogen colonisation and suppresses cell death triggered by  
 356 perception of the *P. infestans* PAMP INF1. We show that it does this through its  
 357 interaction with the potato BTB/POZ domain family protein StNRL1, with which it

358 interacts in the cytoplasm and at the plant plasma membrane, as silencing *NRL1*  
359 compromises the ability of Pi02860 to suppress INF1-mediated cell death. In  
360 contrast, *NRL1* silencing did not attenuate AVR3a suppression of INF1-triggered cell  
361 death, consistent with this effector acting through an alternative host target, the E3  
362 ubiquitin ligase CMPG1 (Bos et al 2010). The observation that AVR3a retains its  
363 ability to attenuate INF1-mediated cell death on *NRL1* VIGS plants may suggest that  
364 AVR3a acts downstream of Pi02860 in suppressing this immune response.

365 Recently, functional redundancy in the *P. infestans* effector repertoire was  
366 highlighted by the demonstration that 8 out of 33 tested RXLR effectors were able to  
367 suppress early transcriptional responses to the bacterial PAMP flg22 (Zheng et al  
368 2014). Of these 8 effectors, only 3 acted to suppress MAPK activation following flg22  
369 treatment, indicating that this functional redundancy likely comprises different  
370 modes-of-action by these effectors; involving some acting upstream and others  
371 downstream of MAPK activation (Zheng et al 2014). The demonstration that Pi02860  
372 and AVR3a (Bos et al 2010; Gilroy et al 2011) each suppress INF1-mediated cell  
373 death, but through activity on different targets, further emphasises functional  
374 redundancy comprising diverse effector activities.

375 *NRL1* is a member of a family of proteins that include the functionally characterised  
376 NPH3 and RPT2, which interact with phototropins at the plasma membrane (PM) to  
377 mediate blue light signalling. The BTB/POZ domain in NPH3 promotes association  
378 with Cullin 3 (CUL3), forming a substrate adaptor in a CRL3<sup>NPH3</sup> (for Cullin-RING-  
379 ubiquitin-ligase) complex that targets phototropin phot1 for ubiquitination. High blue  
380 light conditions result in either mono-/multi- or poly-ubiquitination, the latter of which  
381 targets phot1 for degradation by the 26S proteasome, presumably to attenuate  
382 signalling under light-sufficient conditions (reviewed in Liscum et al 2014). Under low  
383 blue light conditions, in contrast, only mono-/multi-ubiquitination of phot1 occurs,  
384 which is necessary to establish phototropic responses (Roberts et al 2011). One of  
385 the consequences of phot1 activation by the combination of its phosphorylation and  
386 mono-/multi-ubiquitination, is its dissociation from the PM to stimulate relocalisation  
387 of PIN proteins from endosomes to the PM, where they facilitate auxin efflux (Liscum  
388 et al 2014). Poly-ubiquitination, targeting phot1 for proteasome-mediated  
389 degradation, would fail to re-localise PIN proteins and thus not stimulate auxin efflux.

390 Auxin is antagonistic to the defence hormone salicylic acid (SA), and increasing  
391 cellular levels of auxin is a strategy employed by numerous pathogens to suppress  
392 immunity (Naseem and Dandekar 2012). It is thus conceivable that Pi02860 could  
393 promote NRL1 activity, thus influencing phot1 levels, and therefore PIN re-  
394 localisation, retaining intracellular auxin levels to antagonise immunity. However, at  
395 this stage the function of the Arabidopsis orthologue of NRL1 is unknown, and it may  
396 not function similarly to NPH3, instead facilitating the ubiquitination and turnover of  
397 other proteins directly associated with immunity. To investigate this possibility,  
398 further work is needed to identify protein partners of StNRL1, including whether it  
399 forms a ubiquitin E3 ligase complex with CUL3.

400 A number of ubiquitin E3 ligases negatively regulate PTI, and these have been the  
401 subject of extensive functional studies. E3 ligases PUB12 and PUB13 both work to  
402 attenuate PTI by ubiquitinating the flg22 receptor FLS2, facilitating its degradation  
403 (Lu et al 2011). Recent yeast-2-hybrid (Y2H) screens have revealed potential co-  
404 regulatory partners and substrates for ubiquitination by PUB13, including  
405 phosphatidylinositol-4 kinase and RABA4B, with which it complexes to negatively  
406 regulate salicylic acid (SA)-mediated defences (Antignani et al 2015), and the ABA  
407 regulator ABI1, a PP2C family member, which is a PUB13 substrate for  
408 ubiquitination and degradation (Kong et al 2015). In addition, PUBs 22, 23, and 24  
409 also suppress immunity. PUB22 attenuates PTI by targeting the exocyst component  
410 exo70B2 for ubiquitination and degradation (Stegmann et al 2012). A further  
411 example of E3 ligases that negatively regulate immunity are the BTB-domain  
412 proteins NPR3 and NPR4, which form complexes with CUL3 to facilitate the  
413 ubiquitination and degradation of the major SA regulator NPR1 in the nucleus (Fu et  
414 al 2012). If NRL1 forms a complex with CUL3 it may represent a further CUL3-based  
415 E3 ligase involved in negative regulation of immunity, albeit one that is predicted to  
416 function outside of the nucleus. Identification of its substrates for ubiquitination will  
417 reveal the mechanism underlying its defence suppression.

418 Whereas transient silencing of *NbNRL1*, using VIGS, accelerated INF1 cell death  
419 and attenuated *P. infestans* leaf colonisation, transient overexpression of StNRL1  
420 resulted in the opposite phenotypes, indicating that, in the absence of the effector  
421 Pi02860, NRL1 is a negative regulator of immunity, and can thus be regarded as a  
422 susceptibility (S) factor. The term S factor has been coined to describe proteins with

423 a wide range of activities from cell wall alterations, to proteins that directly suppress  
424 or antagonise immunity, to those that provide metabolic changes of benefit to  
425 pathogen growth (van Schie and Takken, 2014). Many have been defined as such  
426 due to reduced pathogen colonisation when they are disabled, and/or increased  
427 disease development when they are overexpressed. Few such proteins have been  
428 demonstrated to be targeted by pathogen effectors. Examples include the *SWEET*  
429 genes that are induced by *Xanthomonas* TAL effectors, contributing to sugar efflux to  
430 provide pathogen nutrition (Chen et al. 2010); the *Pseudomonas* effector AvrB which  
431 mediates the phosphorylation and activation of MPK4, a negative regulator of PTI  
432 (Cui et al., 2010); and more recently, the *P. infestans* effector Pi04089, which targets  
433 and stabilises a KH RNA binding protein, StKRBP1, overexpression of which  
434 enhances susceptibility (Wang et al., 2015). Here we show that the target of  
435 Pi02860, NRL1, is an S factor that directly or indirectly suppresses PTI, in the form of  
436 INF1-mediated cell death. Future work will focus on how Pi02860 supports or  
437 promotes NRL1 activity, and in identifying the substrates and partner proteins of  
438 NRL1, and how it acts to enhance late blight susceptibility. Understanding how *P.*  
439 *infestans* can use endogenous host regulatory proteins and processes that may  
440 naturally undermine immunity will reveal novel means to control this pathogen.  
441 Further studies on NRL1, as a negative regulator of immunity, will indicate the  
442 mechanisms by which plants govern cross-talk between biotic stress responses and  
443 other cellular processes in an attempt to balance and allocate resources.

444

## 445 **Materials & Methods**

### 446 **Vector construction**

447 *Phytophthora infestans* putative RXLR effector gene Pi02860 was synthesised by  
448 Genscript with attL sites to generate an entry vector. To make overexpression vector  
449 PRI101-Pi02860 the effector was amplified from *P. infestans* cDNA with primers  
450 containing *Bam*HI and *Nde*I restriction sites and ligated into PRI101 using standard  
451 molecular biology techniques. The potato NPH3/RPT2-like protein StNRL1 coding  
452 sequence was amplified from *S. tuberosum* cDNA with flanking attB sites and PCR  
453 products were recombined into pDONR201 (Invitrogen) to generate entry clones  
454 using Gateway technology (Invitrogen) primer sequences shown in supplemental  
455 Table S1.

456 The effector entry clones were recombined with pDEST32 (for Y2H; Invitrogen),  
457 pB7WGF2 (for N-terminal EGFP fusion) (Karimi et al., 2002). Modified forms of  
458 pB7WGF2 with either an NES signal derived from PKI: amino acid sequence  
459 LALKLAGLDIN (Wen et al., 1995) or an NLS signal derived from SV40 T antigen:  
460 amino acid sequence PKKKRKV (Kalderon et al., 1984) added to the N-terminus of  
461 the GFP were created. The effector entry clones were also recombined with pCL112  
462 (for N-terminal YN fusion) or pCL113 (for N-terminal YC fusion) for BiFC (Bos et al.,  
463 2010) and pGWB18 (for N-terminal tagging with the cMyc epitope), (Nakagawa et  
464 al., 2007).

### 465 **Potato transformation**

466 *Agrobacterium* containing overexpression vector PRI101-Pi02860 was used to  
467 transform microtuber discs of the potato cultivar E3 (Si et al., 2003; Tian et al.,  
468 2015). Positive lines were first screened on differential medium (3% MS+0.2 mg l<sup>-1</sup>  
469 IAA + 0.2 mg l<sup>-1</sup> GA3 + 0.5 mg l<sup>-1</sup> 6-BA + 2 mg l<sup>-1</sup> ZT+75 mg l<sup>-1</sup> Kan+200 mg l<sup>-1</sup>  
470 Cef, pH 5.9) and then transferred to root generation medium (3% MS+50 mg l<sup>-1</sup> kan  
471 + 400 Cef mg l<sup>-1</sup>, pH 5.9). The presence and expression level of the transgene was  
472 confirmed by semi-quantitative PCR (primers are shown in supplemental Table S1).

473

474

475 **Plant production and maintenance**

476 *Nicotiana benthamiana* and *Solanum tuberosum* overexpression (OE) lines were  
477 grown in glasshouses in 16h days at 22°C. Supplementary light was provided when  
478 the ambient light dropped below 200W / m<sup>2</sup> and shading when it was above 450W /  
479 m<sup>2</sup>. Approximately five week old *N. benthamiana* and 7 week old *S. tuberosum*  
480 plants were used.

481 **Agroinfiltration and infection assays**

482 *A. tumefaciens* strain AGL1 transformed with vector constructs were grown overnight  
483 in YEB medium containing selective antibiotics at 28 °C, pelleted, resuspended in  
484 infiltration buffer (10 mM MES, 10 mM MgCl<sub>2</sub> and 200 µM acetosyringone) and  
485 adjusted to the required OD600 before infiltration into *N. benthamiana* leaves  
486 (generally 0.005 to 0.01 for imaging purposes, 0.002 for BiFC, 0.1 for infection  
487 assays and 0.5 for HR assays). For co-expression agrobacterial cultures carrying the  
488 appropriate vector constructs were mixed prior to infiltration.

489 *Phytophthora infestans* strain 88069 was used for plant infection and was cultured  
490 on Rye Agar at 19 °C for 2 weeks. Plates were flooded with 5ml H<sub>2</sub>O and scraped  
491 with a glass rod to release sporangia. The resulting solution was collected in a falcon  
492 tube and sporangia numbers were counted using a haemocytometer and adjusted to  
493 30000 sporangia/ ml, 10µl droplets were inoculated onto the abaxial side of leaves of  
494 intact *N. benthamiana* plants stored on moist tissue in sealed boxes. For VIGSed  
495 plants the average lesion diameter was measured and compared to the GFP control  
496 plants. *Agrobacterium tumefaciens* Transient Assays (ATTA) in combination with *P.*  
497 *infestans* infection were carried out as described (McLellan et al., 2013).

498 **Confocal imaging**

499 *N. benthamiana* cells were imaged at 2 dpi using Leica TCS SP2 AOBS, Ziess 710  
500 or Nikon A1R confocal microscopes with Leica HCX PL APO lbd.BL 63x/1.20 W and  
501 L 40x/0.8, Zeiss PL APO 40x/1.0 or Nikon 60x/ water dipping objectives. GFP was  
502 excited by the 488 nm line of an argon laser and emissions were detected between  
503 500 and 530 nm. The pinhole was set to 1 airy unit for the longest wavelength  
504 fluorophore. Single optical section images and z-stacks were collected from leaf cells  
505 expressing low levels of the protein fusions to minimise the potential artefacts of

506 ectopic protein expression. Images were projected and processed using the Leica  
507 LCS, Zen 2010 or NIS-Elements software packages. Subsequent image processing  
508 for Figure generation was conducted with Adobe Photoshop CS2 and Adobe  
509 Illustrator.

### 510 **Yeast-two-hybrid and Co-immunoprecipitation**

511 A Y2H screen with pDEST32-Pi02860 was performed as described in (McLellan et  
512 al., 2013) using the Invitrogen ProQuest system. The full-length coding sequence of  
513 the candidate interacting prey sequence, *StNRL1* (accession Sotub02g031050.1.1)  
514 was cloned and re-tested with pDest32-Pi02860 and pDEST32-PiAvr2 as a control  
515 to rule out the possibility that the observed reporter gene activation had resulted from  
516 interactions between the prey and the DNA binding domain of the bait construct or  
517 DNA binding activity of the prey itself.

518 *A. tumefaciens* strain GV3101 containing the fusion protein constructs were grown  
519 overnight in YEB medium containing selective antibiotics at 28 °C, pelleted,  
520 resuspended in infiltration buffer (10 mM MES, 10 mM MgCl<sub>2</sub> and 200 μM  
521 acetosyringone) and adjusted to an OD<sub>600</sub> of 1.0 before infiltration into *N.*  
522 *benthamiana* leaves. Forty eight hours post infiltration samples were taken and  
523 proteins extracted. GFP tagged Pi02860/ PiAvr2 fusions were immunoprecipitated  
524 using GFP-Trap®-M magnetic beads (Chromotek GmbH). The resulting samples  
525 were separated by PAGE and Western blotted. Immunoprecipitated GFP fusions and  
526 co-immunoprecipitated c-Myc fusions were detected using appropriate antisera  
527 (Santa Cruz Biotechnology, UK).

### 528 **Virus Induced Gene Silencing**

529 Virus induced gene silencing (VIGS) constructs were made by cloning a 250 bp PCR  
530 fragment shared by *NbNRL1a* (accession NbS00004529g0005.1) and *NbNRL1b*  
531 (accession NbS00009404g0009.1) from *N. benthamiana* cDNA and cloning into  
532 pBinary Tobacco Rattle Virus (TRV) vectors (Liu et al., 2002) between *HpaI* and  
533 *EcoRI* sites in the antisense orientation. BLAST analysis of this sequence against  
534 the *P. infestans* genome  
535 ([http://www.broadinstitute.org/annotation/genome/phytophthora\\_infestans/ToolsIndex.html](http://www.broadinstitute.org/annotation/genome/phytophthora_infestans/ToolsIndex.html))  
536 did not reveal any matches that could initiate silencing in the pathogen. A

537 TRV construct expressing GFP described previously was used as a control  
538 (McLellan et al., 2013). Primer sequences are shown in Supplementary Table1. The  
539 two largest leaves of four leaf stage *N. benthamiana* plants were pressure infiltrated  
540 with LBA4404 *A. tumefaciens* strains containing a mixture of RNA1 and each NRL  
541 VIGS construct or the GFP control at OD600 = 0.5 each. Plants were used for  
542 assays or to check gene silencing levels by qRT-PCR 2-3 weeks later.

#### 543 **Gene expression assay**

544 RNA was extracted using a Qiagen RNeasy Kit with on the column DNA digestion  
545 steps according to the manufacturer's instructions. First strand cDNA was  
546 synthesised from 2µg of RNA using Superscript II RNase HReverse Transcriptase  
547 (Invitrogen) according to manufacturer's instructions. Realtime qRT-PCR reactions  
548 were performed using Power SYBR Green (Applied Biosystems) and run on a  
549 Chromo4 thermal cycler (MJ Research, UK) using Opticon Monitor 3 software.  
550 Primer pairs were designed outside the region of cDNA targeted for silencing  
551 following the manufacturer's guidelines. Primer sequences in Supplementary Table1.  
552 Detection of real-time RT-PCR products, calculations and statistical analysis were  
553 performed as previously described (McLellan et al. 2013).

#### 554 **Supplementary Information**

555 **Figure S1.** Transgenic potato lines overexpress Pi02860.

556 **Figure S2.** Immunoblots showing stability of the Pi02860 GFP fusions for re-  
557 localisation experiments.

558 **Figure S3.** Alignment of *Arabidopsis thaliana* (At), *N. benthamiana* (Nb) and *S.*  
559 *tuberosum* (St) NRL1 sequences.

560 **Figure S4.** Pi02860 interaction with NRL1 largely occurs at the plant plasma  
561 membrane.

562 **Figure S5.** Western blots showing stability of the different split YFP and GFP  
563 constructs used.

564 **Figure S6.** Silencing levels and plant phenotypes for VIGS of NRL1 in *N.*  
565 *benthamiana*.

566 **Figure S7.** Stability of StNRL1 is not altered by Pi02860.

567 **Table S1.** Primers used in this study.

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575 Consortium for helpful discussions during this work.

576

## 577 **Figure Legends**

578 **Figure 1. Transient or stable overexpression of Pi02860 enhances *P. infestans***  
579 **colonisation.** A, Graph shows a significant increase ( $p < 0.001$ ,  $n = 94$ ) in *P. infestans*  
580 lesion diameter following transient expression in *N. benthamiana* of GFP-Pi02860  
581 compared to a free GFP control. Trypan blue stained leaf image showing the extent  
582 of *P. infestans* colonisation with GFP-Pi02860 or free GFP. B, Graph shows that  
583 transgenic potato lines overexpressing Pi02860 (OE Pi02860-1 and OE Pi02860-2)  
584 allow a significant increase (ANOVA,  $p < 0.002$ ,  $n = 83$ ) in *P. infestans* lesion diameter  
585 compared to the potato cv. E3 control. C, Graph shows transgenic potato lines  
586 overexpressing Pi02860 allow a significant increase ( $p < 0.001$ ,  $n = 64$ ) in *P. infestans*  
587 sporulation compared to the E3 control. D, Leaf images taken under UV light show  
588 an increase in *P. infestans* lesions in transgenic potato lines overexpressing Pi02860  
589 compared to the E3 control. Lower case letters on graphs denote statistically  
590 significant differences determined by one-way ANOVA with pairwise comparisons  
591 performed with the Holm-Sidak method. Results are the combination of at least two  
592 independent biological replicates, error bars show standard error.

593 **Figure 2. Pi02860 suppresses INF1 but not Cf4-Avr4 HR.** A, Graph shows transient  
594 overexpression of GFP-Pi02860 compared to free GFP in *N. benthamiana* can  
595 significantly suppress the HR ( $p < 0.001$ ,  $n = 11$ ) triggered by the elicitor INF1 to a  
596 similar extent as the control GFP-Avr3a. B, Representative leaf image showing INF1  
597 HR at 6 dpi, following co-expression with constructs as indicated. C, Graph shows  
598 transient overexpression of GFP-Pi02860 or free GFP in *N. benthamiana* show no  
599 significant difference ( $p > 0.1$ ) in HR triggered by Cf4-Avr4 whereas GFP-Avr3a  
600 significantly suppresses this HR ( $p < 0.001$ ,  $n = 13$ ). D, Representative leaf image  
601 showing Cf4-Avr4 HR at 6dpi, following co-expression with constructs as indicated.  
602 Lower case letters on graphs denote statistically significant differences by one way  
603 ANOVA with pairwise comparisons performed with the Holm-Sidak method. Error  
604 bars show standard error.

605 **Figure 3. Cytoplasmic localisation of GFP-Pi02860 is important for *P. infestans***  
606 **virulence and INF1 HR suppression phenotypes.** A, Confocal images showing  
607 that GFP-Pi02860 is localised in the cytoplasm and nucleus, while  $_{NES}$ GFP-Pi02860  
608 is greatly reduced in nuclear fluorescence, and  $_{NLS}$ GFP-Pi02860 is concentrated in

609 the nucleus and reduced in the cytoplasm. Upper panels show stacked projections of  
610 single cells, while the lower panels show single slice images of the nuclei. Scale bars  
611 is 10  $\mu$ M. B, Graph shows GFP-Pi02860 and  $_{NES}$ GFP-Pi02860 expression leads to a  
612 statistically significant increase ( $p < 0.001$ ,  $n = 68$ ) in *P. infestans* lesion diameter  
613 compared to free GFP, whereas  $_{NLS}$ GFP-Pi02860 shows an intermediate phenotype.  
614 C, Representative leaf image showing *P. infestans* lesions following overexpression  
615 of each construct, as indicated, in *N. benthamiana*. D, Graph shows GFP-Pi02860  
616 and  $_{NES}$ GFP-Pi02860 co-expression with INF1 leads to a statistically significant  
617 decrease ( $p < 0.001$ ,  $n = 41$ ) in HR compared to free GFP, whereas  $_{NLS}$ GFP-Pi02860  
618 shows an intermediate phenotype. E, Representative leaf image showing INF1 HR  
619 following co-expression with each construct, as indicated, in *N. benthamiana*. Lower  
620 case letters on graphs denote statistically significant differences by one way ANOVA  
621 with pairwise comparisons performed with the Holm-Sidak method. Results are the  
622 combination of three independent biological replicates, each with 18 infiltration  
623 zones. Error bars show standard error.

624 **Figure 4. Pi02860 interacts with the potato BTB/POZ domain protein StNRL1 in**  
625 **yeast-2-hybrid and immunoprecipitation assays.** A, Yeast co-expressing StNRL1  
626 with Pi02860 grew on -histidine (-HIS) medium and yielded  $\beta$ -galactosidase (B-Gal)  
627 activity, while those co-expressed with the control PiAvr2 did not. The +HIS control  
628 shows all yeast were able to grow in the presence of histidine. B,  
629 Immunoprecipitation (IP) of protein extracts from agroinfiltrated leaves using GFP-  
630 Trap confirmed that cMyc-tagged NRL1 specifically associated with GFP-Pi02860  
631 and not with the GFP-PiAvr2 control. Expression of constructs in the leaves is  
632 indicated by +. Protein size markers are indicated in kDa, and protein loading is  
633 indicated by Ponceau stain.

634 **Figure 5. GFP-tagged StNRL1 predominantly locates to the plasma membrane**  
635 **and Bi-molecular fluorescence complementation confirms StNRL1 and Pi02860**  
636 **interaction.** A, Single optical slice image across plasma membranes (PM) of two  
637 adjacent cells co-expressing GFP-StNRL1 and the mOrange-LTi PM marker with a  
638 profile across the membranes in the location indicated by the red arrow. The plot of  
639 the profile (right) indicates the majority of the GFP fluorescence (green line) co-  
640 locates with the PM marker (red). B, A comparable profile in a cell co-expressing the  
641 PM marker with un-fused GFP, which is only present in the cytoplasm. C, Graph

642 shows the average number of fluorescent cells per image with YC-StNRL1 + YN-  
643 Pi02860 and YC-StBSL1 + YN-Pi02860 giving significantly more ( $p < 0.001$ ,  $n = 22$ )  
644 reconstitution of YFP fluorescence than when non-interacting effector-interactor pairs  
645 are used. Lower case letters on graphs denote statistically significant differences by  
646 one-way ANOVA with pairwise comparisons performed with the Holm-Sidak method.  
647 Results are the combination of three independent biological replicates; error bars  
648 show standard error. D, Single optical slice image across PMs of two adjacent cells  
649 co-expressing YC-StNRL1 + YN-Pi02860 and the mOrange-LTi PM marker with a  
650 profile across the membranes in the location indicated (arrow). The plot of the profile  
651 (right) indicates the majority of the reconstituted YFP fluorescence (green line) co-  
652 locates with the PM marker (red line). E, A comparable profile in a cell co-expressing  
653 un-fused YFP in the cytoplasm with the PM marker.

654 **Figure 6. Silencing of *NRL1* in *N. benthamiana* compromises *P. infestans***  
655 **infection.** A, Graph shows that silencing of *NRL1* using two independent VIGS  
656 constructs (TRV-NRL V1 and TRV-NRL V2) in *N. benthamiana* significantly reduces  
657 (one-way ANOVA,  $p < 0.001$ ,  $n = 464$ ; significance denoted by lower case letters) *P.*  
658 *infestans* lesion diameter compared to the TRV-GFP control. B, Representative leaf  
659 images stained with trypan blue to show the extent of *P. infestans* leaf colonisation  
660 on plants expressing each VIGS construct, as indicated.

661 **Figure 7. Silencing of *NRL1* in *N. benthamiana* accelerates INF1 HR and**  
662 **reduces the ability of Pi02860 to attenuate INF1 HR.** A, Graph shows a significant  
663 increase ( $p < 0.001$ ,  $n = 30$ ) in INF1 HR in TRV-NRL V1 and TRV-NRL V2 plants  
664 compared to the TRV-GFP control at 5 dpi but not at 6 dpi. B, Graph shows no  
665 significant changes ( $p > 0.15$ ,  $n = 30$ ) in Cf4-Avr4 HR between TRV-NRL V1, TRV-NRL  
666 and TRV-GFP at 5 or 6dpi. C, Graph shows that GFP-Pi02860 expression is  
667 significantly less able to inhibit INF1 HR ( $p > 0.03$ ,  $n = 39$ ) in TRV-NRL V1 and TRV-  
668 NRL V2 plants compared to TRV-GFP at 6dpi, while having no significant effect on  
669 GFP-Avr3a INF1 HR suppression. Lower case letters on graphs denote statistically  
670 significant differences by one-way ANOVA with pairwise comparisons performed  
671 with the Holm-Sidak method. Results are the combination of three independent  
672 biological replicates. Error bars show standard error.

673 **Figure 8. *NRL1* is downregulated by both *P. infestans* infection and PAMP treatment**  
674 **while overexpression of *NRL1* enhances *P. infestans* leaf colonisation and**  
675 **suppresses INF1-triggered cell death.** A, Graph shows relative expression levels of  
676 *NbNRL1a* and *NbNRL1b* and *NbWRKY7* and *NbWRKY8* in response to *P. infestans*  
677 infection. B, Graph shows relative expression levels of *NbNRL1a* and *NbNRL1b* and  
678 *NbWRKY7* and *NbWRKY8* in response to *P. infestans* Culture Filtrate (CF) treatment. C,  
679 Graph shows that overexpression of GFP-Pi02860 and GFP-StNRL1 significantly  
680 increases ( $p < 0.001$ ,  $n = 106$ ) *P. infestans* lesion size compared to free GFP.  
681 Representative leaf image showing *P. infestans* lesions following overexpression of  
682 each construct, as indicated, in *N. benthamiana*. D, Graph shows overexpression of  
683 GFP-Pi02860 and GFP-StNRL1 significantly decrease ( $p < 0.001$ ,  $n = 44$ ) INF1 HR  
684 compared to free GFP. Representative leaf image showing INF1 HR with  
685 overexpression of each construct in *N. benthamiana*. E, Graph shows co-expression  
686 of GFP-Pi02860 or GFP-StNRL1 have no significant effect ( $P = 0.325$ ,  $n = 24$ ) on Cf4-  
687 Avr4 HR compared to free GFP. Representative leaf image showing Cf4-Avr4 HR  
688 with overexpression of each construct in *N. benthamiana*. Lower case letters on  
689 graphs denote statistically significant differences by one-way ANOVA with pairwise  
690 comparisons performed with the Holm-Sidak method. Results are the combination of  
691 three independent biological replicates. Error bars show standard error.

692



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