1 # NRG1-mediated recognition of HopQ1 reveals a link between PAMP-

2 # and Effector-triggered Immunity.

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Summary

- Members of the hopQ1/XopQ family of effectors are conserved amongst pathogenic bacteria suggesting an important function in virulence. Therefore, the identification of R proteins recognising members of this family is potentially of high value to achieve broad-spectrum resistance in crops.

- Recent studies demonstrated that HopQ1/XopQ recognition is EDS1-dependant and is mediated by the TIR-NB-LRR protein Roq1. Using our recently described R gene RNAi library, we have investigated the mechanism of HopQ1 recognition and the other components of its signalling network.

- We show that silencing of the CC_R-NB-LRR helper protein N Requirement Gene 1 (NRG1) prevents HopQ1 recognition in *N. benthamiana* and that NRG1 expression confers recognition of HopQ1 and restricts *Pto* DC3000 proliferation in the otherwise susceptible host *Arabidopsis*. Moreover NRG1 expression is briefly but strongly induced at a very early stage of the plant innate response, establishing a strong dependency link between PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI).

- Overall we demonstrate that in addition to Roq1, HopQ1 recognition in *N. benthamiana* requires the CC_R-NB-LRR protein NRG1 and propose a model based on the dual requirement of a CC_R-NB-LRR and a TIR-NB-LRR that could extend beyond HopQ1 detection and possibly be used to recognize other pathogen effectors.

Key words: Effectors, Effector-triggered immunity (ETI), HopQ1, NB-LRR proteins, NRG1, PAMP-triggered immunity (PTI), plant-microbe interaction.

Introduction

In the course of their co-evolution, plants and pathogens have acquired increasingly complex mechanisms of resistance and susceptibility to overcome one another. In addition to physical barriers to prevent non-specific microbial attacks, plants possess two main layers of defence to counter local infections by adapted pathogens. The first layer of defence is activated upon the detection at the plasma membrane of a wide variety of evolutionarily conserved pathogen elicitors, referred to as Pathogen-Associated Molecular Pattern (PAMPs), by specific

54   extracellular receptors (Pattern Recognition Receptors or PRRs). The PAMP-triggered

55   immunity (PTI) leads via a complex signalling cascade to a range of cellular defence responses

56   including Reactive Oxygen Species production, ion transport, or callose deposition. However

57   many gram-negative pathogenic bacteria possess a type III secretion system (T3SS) which

58   allows them to inject virulence factors (effectors) into plant cells specifically to overcome PTI.

59   In turn, plants have developed a second layer of immunity involving intracellular receptors

60   (resistance or R proteins) able directly or indirectly to sense the presence of bacterial

61   effectors. The resulting effector-triggered immunity (ETI) typically leads to a rapid localized

62   programed cell death (PCD), also referred to as Hypersensitive Response (HR), to prevent

63   further progress of the pathogen. The co-evolution of microbes and their host plants

64   (illustrated in the zig-zag model of Jones and Dangl, 2006) stimulates pathogens to constantly

65   modify their repertoire of effectors to evade recognition and gain a fitness edge, and plants

66   to increase their R protein diversity to regain resistance, leading to increasingly complex

67   mechanisms of resistance and susceptibility.

68   Most of the R proteins identified to date are Nucleotide-Binding Leucine-Rich Repeat-

69   containing proteins (NB-LRR) and usually include either a Coiled-Coil (CC) or a Toll Interleukin-

70   1 receptor (TIR) domain in their N-termini. Activation of the PCD by NB-LRR is dependent on

71   the well-conserved protein SUPPRESSOR OF G2 ALLELE OF SKP1 (SGT1) (Shirasu, 2009), which

72   forms a complex with the HEAT SHOCK PROTEIN 90 (HSP90) and REQUIRED FOR MLA12

73   RESISTANCE 1 (RAR1) proteins to mediate stability of NB-LRR proteins (Lu *et al.*, 2003; Boter

74   *et al.*, 2007; Zhang *et al.*, 2010). Other components of the ETI signalling pathway have been

75   identified, such as ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) or NONRACE-SPECIFIC

76   DISEASE RESISTANCE 1 (NDR1), specifically required for the TIR-NB-LRR (abbreviated to TNL

77   thereafter) and most of the CC-NB-LRR (abbreviated to CNL thereafter)-mediated ETI,

78   respectively (Century *et al.*, 1995; Parker *et al.*, 1996; Aarts *et al.*, 1998; Zhu *et al.*, 2011;

79   Wagner *et al.*, 2013).

80   Phytopathogenic bacterial strains are often defined by their host specificity, which is largely

81   shaped by their repertoire of effectors. Host resistance specified by major resistance genes is

82   typically conferred by direct or indirect recognition of an effector and is sometimes easily

83   overcome by loss of that effector. It has been suggested that resistance derived from non-

84   host species comprises various mechanism, therefore leading to a more complex and durable

85   resistance (Mysore & Ryu, 2004; Gill *et al.*, 2015). Consequently there has been a growing

86   interest in recent years in identifying components of resistance to pathogens from non-host

87   species as they are believed to provide potentially more durable R genes than those

88   originating from host species (Lee *et al.*, 2016). However, the distinction between host and

89   non-host resistance may not be as substantial as first proposed and led Schulze-Lefert and

90   Panstruga (2011) to propose that they differ mainly in the relative contributions from NB-LRR

91   and PRR recognition components.

92   *Pseudomonas syringae pv. tomato* (*Pto*) DC3000 causes bacterial speck disease in tomato. It

93   can also infect the model plant Arabidopsis and induces an HR in *Nicotiana benthamiana* (*Nb*).

94   A recent study demonstrated that deletion of a single effector of its repertoire, HopQ1, is

95   enough for *Pto* to cause disease in *Nb* (Wei *et al.*, 2007). HopQ1, like its homolog in

96   *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) XopQ, is the sole effector, among the

97   repertoire of these bacteria to be recognized in both *Nb* and *Nicotiana tabacum* (*Nt*) (Wei *et*

98   *al.*, 2007; Adlung *et al.*, 2016). XopQ recognition within the *Solanaceae* family is largely

99   restricted to the *Nicotiana* genus. HopQ1 and XopQ share significant homology and are

100 functionally interchangeable. Since their recognition in *Nb* is EDS1-dependent (Adlung *et al.*,

101 2016; Adlung & Bonas, 2017), both effectors are likely to be recognized by a conserved

102 mechanism within the *Nicotiana* genus involving a TNL R gene. Indeed the TNL protein Roq1

103 was recently identified as required to mediate HopQ1/XopQ recognition in *Nb* (Schultink *et*

104 *al.*, 2017). Given the broad distribution of hopQ1/XopQ homologs within bacterial

105 phytopathogens, including *Pseudomonas*, *Xanthomonas* and *Ralstonia* (RipB), the

106 identification of R proteins able to recognise these effectors is potentially valuable to achieve

107 resistance to a wide range of plant pathogens.

108 Previously we reported a novel approach to identify R genes involved in the recognition of

109 effectors of interest triggering ETI in *Nb* (Brendolise *et al.*, 2017). Using this approach we

110 demonstrate that HopQ1-triggered HR is not only dependent on Roq1 but also requires NRG1,

111 a CNL R gene previously implicated in *Tobacco Mosaic Virus* (TMV) resistance in *Nt* (Peart *et*

112 *al.*, 2005). Here, we show that silencing of NRG1 promotes *Pto* proliferation in *Nb* and that

113 NRG1 expression confers recognition of HopQ1 and can restrict *Pto* proliferation in the

114 susceptible host Arabidopsis. We also show that the expression of NRG1 is induced at the

115 level of mRNA in response to PTI and discuss how this leads to an interesting new connection

116   between PTI and HopQ1-mediated ETI that reveals a previously undetected degree of

117   coordination between these two layers of defence response.

Materials and Methods

119   **Plant material and transformation**

120   *N. benthamiana* plants were grown in glasshouse at 22 to 24°C with natural light combined

121   with supplemental lighting to extend the day length to 16 h**.** Arabidopsis plants (Columbia-0)

122   were grown at 25°C in growth cabinets with 10 h photoperiod. Plants were transformed

123   following the previously described floral dip method (Clough & Bent, 1998). Seeds were

124   harvested and sterilized using a solution of 70% ethanol/0.05% triton and grown on ½ MS

125   selecting media containing 50 mg/L kanamycin.

126   **Cloning and constructs**

127   The *HOPQ1* coding sequence was amplified from *Pto* DC3000 using primers FW 5'-

128   CACCATGCATCGTCCTATCACCGCAG-3' and RV 5'-TCAATCTGGGGCTACCGTCGACTG-3' and

129   inserted in the pENTR/SD/D-TOPO vector (Invitrogen) following the manufacturer's protocols

130   to generate the pENTR/SD/D-HopQ1 entry clone, which was recombined by Gateway LR

131   reaction into the pHEX2 binary vector (Hellens *et al.*, 2005). Similarly, the pENTR-GUS entry

132   clone (Invitrogen) was recombined by Gateway LR reaction into pHEX2 to generate the

133   pHEX2-GUS control.   Hp#26, hp#27 and pTKO2-u135 have been described previously

134   (Brendolise *et al.*, 2017). All the other individual fragments from hp#26 and hp#27 were

135   cloned following the procedure described for pTKO2-u135, consisting of an In-Fusion HD

136   cloning reaction (Clontech) into the pENTR/SD/D-TOPO vector to generate an entry clone,

137   followed by an LR Gateway reaction into the pTKO2 destination vector (Brendolise *et al.*,

138   2017). The TRV1 and the Gateway-enabled TRV2 (pYL279) used for gene silencing in *N.*

139   *benthamiana* were previously described (Liu *et al.*, 2002). The pENTR/SD/D-u135 entry clone

140   used to generate the pTKO2-u135 construct was recombined by LR reaction into pYL279 to

141   generate the TRV2-u135 construct.

142   **Transient expression and RNAi library screen**

143   Transient expression and the RNAi library screen in *N. benthamiana* were described

144   previously (Brendolise *et al.*, 2017). In brief, RNAi hairpin constructs, or pTKO2 empty vector,

145   were agro-infiltrated (OD_{600} of 0.2) in leaves of 3-weeks old plants and the infiltrated area was

146   marked. The HopQ1 construct or the GUS control were agro-infiltrated 48h later in the pre-

147   infiltrated area. Photographs were taken 4 days after HopQ1 infiltration. For biolistic

148   transient expression, 5 mg of gold particles (1.0 µm diameter, BioRad) were combined with 5

149   µg of GFP plasmid DNA pRT99-GFP (Siemering *et al.*, 1996) and 20 µg of either test DNA (e.g.,

150   HopQ1) or control DNA (pRT99-GUS) (Topfer *et al.*, 1988), treated with 20 µL of 0.1 M

151   Spermidine solution followed by 50 µL of 2.5 M CaCl2 and placed on ice. Excess supernatant

152   was removed to a final volume of 35 µL. Bombardment was performed on well-expanded

153   leaves of *N. benthamiana* and Arabidopsis using a particle inflow gun made by KiwiScientific

154   (Levin, NZ) based on the design of Finer and colleagues (Finer *et al.*, 1992). For each

155   bombardment six target leaves were shot on the abaxial side under a metal mesh screen (100

156   µm) using 5 µL of the particle preparation and the following conditions: carrier to target shelf

157   distance 14 cm (Arabidopsis) or 12 cm (*N. benthamiana*), helium pressure 300 Kpa, pulse time

158   30 ms, chamber vacuum -90 Kpa. Leaves were incubated in the light at room temperature for

159   24 h or 48 h and the number of GFP cells was counted with a fluorescence stereomicroscope

160   (Leica Microsystems M205FA) equipped with a GFP filter set.

161   **VIGS**

162   TRV2-u135 was transformed into GV3101 *Agrobacterium tumefaciens.* Bacteria were grown

163   for two days on LB plates with appropriate antibiotics then resuspended in infiltration buffer

164   (10 mM $MgCl_2$, 10 mM MES, 150 µM acetosyringone). Strains containing TRV2-u135 or TRV2

165   empty vector were mixed with the strain containing TRV1 (1:1 ratio to a final OD_{600} of 0.5 each

166   and kept for 2 h at room temperature in the dark. Two-week-old *N. benthamiana* plants were

167   then infiltrated in one leaf with a 1 mL needleless syringe. Plants were grown for 3 weeks in

168   a culture chamber before use.

169   **Plant inoculation and Bacterial growth quantification**

170   *N. benthamiana* leaves were infiltrated with a bacterial suspension of *Pto* DC3000 ranging

171   from $5x10^5$ cfu/mLto 10^7 cfu/mL as indicated. The best visual difference of disease lesions

172   between NRG1-silenced and control plants were obtained with $5x10^6$ cfu/mL and 10^7 cfu/mL

173   inoculum concentrations (Supporting Information Fig. S1). Immediately after infiltration and 72

174   hours after, 6 leaf discs of 7 mm diameter were collected from five independent patches

175   inoculated at $5x10^6$ cfu/mL, and pulverized with glass beads using a mill grinder and

176   resuspended in water. Dilutions 10^{-3} and 10^{-5} were then plated with an easySpiral® instrument

177   (Interscience) and counted according to the manufacturer recommendations. For each

178   Arabidopsis transgenic line, six leaf discs of 6 mm diameter were collected and surface

179   sterilized using a 0.9% bleach solution. Leaf discs were inoculated by flooding for 3min in a

180   bacterial suspension of *Pto* DC3000 at 10^5 cfu/mL supplemented with 0.025% Silwet L-77.

181   Bacterial suspension was drained and leaf discs were maintained for 3 days on solid ½ MS

182   media. Discs were surface sterilized and pulverized with glass beads in 200 μL MgCl2 buffer

183   using the TissueLyser II system (QIAGEN). Serial dilutions were spotted onto KB media

184   supplemented with rifampicin 25 mg/mL and the number of colonies was evaluated after 48

185   h of incubation at room temperature.

186   **Ion leakage quantification**

187   For each treatment, sixteen 3.5 mm discs were harvested from the agro-infiltrated area of

188   four independent *N. benthamiana* leaves and washed in distilled water for 2h. Four discs were

189   placed in 2mL of water and conductivity was measured over time using a Laquatwin

190   conductivity meter (Horiba). The standard errors of the means were calculated from four

191   replicates.

192   **qPCR**

193   RNA was extracted from Arabidopsis *or N. benthamiana* leaf tissue using the Spectrum Plant

194   Total RNA kit (Sigma–Aldrich) according to the manufacturer's instructions. The RNA was

195   quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA) and

196   quality was evaluated on a 1% agarose gel. DNase treatment and first-strand cDNA synthesis

197   were carried out using the QuantiTect Reverse Transcription Kit (QIAGEN) following the

198   manufacturer's instructions. qRT-PCR amplification was carried out on a LightCycler® 480

199   Real-Time PCR System (Roche) using the LightCycler 480 SYBR Green I Master Mix (Roche)

200   and analysed with the LightCycler 480 software version 1.5. Amplification efficiency of each

201   gene was calculated from the amplification of serial dilutions of the cDNA of the

202   corresponding gene. For the VIGS experiments, RNA was isolated from six leaf discs (7mm

203   diam.) samples using NucleoSpin® RNA kit (Macherey Nagel) and the DNAse treatment was

204   performed using the TURBO DNA-free kit (Invitrogen). Real time qPCR amplification was

205   performed with Transcriptor Reverse Transcriptase (Roche) with random hexanucleotides

206   primers on 2 μg RNA. qRT-PCR was done on a LightCycler 480 using SYBR Green I Master Mix

207   (Roche). Sequences of all the primers are provided in Table S1.

208   **Data Availability**

209   All data generated in this study are included in this article (and its supplemental information

210   file). Material is available upon reasonable request.

Results

211

212   **NRG1 is required for HopQ1 recognition in *N. benthamiana***

213   *Pto* DC3000 triggers an HR in *Nb* and it was shown that HopQ1 is the sole *Pto* DC3000 effector

214   recognised in *Nb* (Wei et al., 2007). We developed a new approach to identify R genes

215   involved in the recognition of an effector of interest triggering an HR in *Nb* (Brendolise *et al.*,

216   2017). This approach is based on the genome-wide identification of most of the *Nb* R genes

217   and their systematic silencing using an R gene RNAi library. HopQ1 was used to screen the 47

218   constructs of our R gene RNAi library following the methodology previously described. Two

219   hairpin constructs, hp#26 and hp#27, were identified as being able to reduce HopQ1-

220   triggered HR significantly (Fig. 1a). To reduce the total number of constructs in the library,

221   hairpins were initially designed to produce multi-gene silencing by concatenation of several

222   fragments each targeting independent genes. Each of the component fragments of hairpin

223   constructs hp#26 and hp#27 were sub-cloned individually in the hairpin vector and their

224   ability to reduce the HopQ1-triggered HR was tested following the same procedure.

225   Fragments u135 and u111 from hairpins hp#26 and hp#27 respectively were identified as the

226   fragments responsible for the reduction of HopQ1-triggered HR (Fig. 1b, c). Fragments u135

227   and u111 target the NRG1 and NRG2 genes respectively, with 100% identity to their targets.

228   NRG1 is a CNL R gene previously shown to be required for TMV resistance in *Nb* (Peart *et al.*,

229   2005). NRG2 shares 86% homology with NRG1 and was previously shown to be a pseudogene

230   truncated in its 5' terminal end. Consequently the effect observed with hp#27 is likely to be

231   due to the silencing of NRG1, given that u111 shares 92.5% identity to NRG1 over the 120 bp

232   of the fragment (Fig. 1d). This was confirmed by the measurement of the NRG1 expression in

233   *Nb* leaves three days after infiltration of the u111 and u135 hairpins. Both hairpins were able

234   to reduce NRG1 expression significantly (Fig. 1e). Interestingly NRG2 transcript was also

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235   detected, however the minor decrease in NRG2 measured in the presence of the two hairpins

236   was not statistically significant (Fig. 1e).

237   **NRG1 silencing promotes *Pto* DC3000 proliferation in *N. benthamiana***

238   Previous studies demonstrated that deletion of HopQ1 allows *Pto* DC3000 to grow to a higher

239   titre and cause disease in *Nb* (Wei *et al.*, 2007). To confirm the requirement of NRG1 in HopQ1

240   recognition, we investigated the effect of NRG1 silencing on the ability of *Pto* DC3000 to grow

241   in *Nb*. *Agrobacterium* pre-infiltration in *Nb* is known to inhibit the *Pseudomonas* T3SS and its

242   ability to cause disease (Chakravarthy *et al.*, 2010). Consequently we were not able to use

243   *Agrobacterium*-mediated expression of the u135 hairpin construct to assess silencing of

244   NRG1. Instead NRG1 was silenced by Virus Induced Gene Silencing (VIGS) using the same

245   fragment as in the u135 hairpin construct. Two week-old *Nb* seedlings were infiltrated with

246   the TRV1 and TRV2-u135 constructs (or the corresponding TRV2 empty vector), after 2 weeks

247   *Pto* DC3000 was inoculated in newly formed leaves and symptoms and bacterial growth were

248   measured. Stronger disease lesions were visible in NRG1-silenced plants than in TRV2 control

249   plants (Fig. 2a and Supporting Information Fig. S1); moreover, *Pto* DC3000 growth measured in

250   these plants was significantly higher than in the control plants (Fig. 2b). qPCR analysis of the

251   corresponding infiltrated patches confirmed that NRG1 expression was reduced in plants

252   expressing the TRV2-u135 construct (Fig. 2c).

253   **Expression of NRG1 in Arabidopsis confers recognition to HopQ1 and restricts *Pto***

254   **DC3000 growth *in planta***

255   *Pto* DC3000 is pathogenic in Arabidopsis (Whalen *et al.*, 1991). Two NRG1 orthologs have been

256   identified in Arabidopsis (AtNRG1.1, AtNRG1.2) based on sequence similarity (53 % and 55 %

257   similarity to NbNRG1 respectively) (Collier *et al.*, 2011). However, the expression of HopQ1 in

258   Arabidopsis does not trigger any HR (Li *et al.*, 2013a; Hann *et al.*, 2014), suggesting that

259   AtNRG1.1 and AtNRG1.2 are not fully functional homologs of NRG1 and/or able to recognise

260   HopQ1.

261   To test whether NRG1 can confer recognition of HopQ1 in Arabidopsis, several lines of stable

262   transformants expressing a *35S:NbNRG1* construct were generated. HopQ1 together with a

263   GFP reporter were transiently expressed in these lines using biolistic delivery and the resulting

264   fluorescence was quantified. HR was evaluated by quantifying a reduction of the GFP signal

265   resulting from cell death. HopQ1 expression induced a significant reduction of the GFP

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266   reporter signal in three of the five NRG1-expressing lines tested (Fig. 3a). Conversely HopQ1

267   expression did not induce an HR in any of the GUS control lines (Fig. 3a and Supporting

268   Information Fig. S2). The effector HopAS1 known to trigger a strong HR in Arabidopsis

269   Columbia-0 (Sohn *et al.*, 2012) was used as an HR positive control. As expected HopAS1

270   expression induced a strong reduction of the GFP signal in both the NRG1 and the GUS lines.

271   Interestingly, NRG1 expression level in the transformant lines inversely correlated with the

272   GFP signal measured (Fig. 3b); indeed the two lines showing a non-significant reduction of the

273   GFP signal also had the lowest level of expression of NRG1.

274   To further assess the NRG1 effect, we tested whether NRG1 expression in Arabidopsis

275   conferred resistance to *Pto* DC3000. Leaf discs from three NRG1 expressing lines were flood-

276   inoculated with *Pto* DC3000 and bacterial growth was measured 4 days later. The three NRG1-

277   expressing lines tested showed a significant decrease in bacterial count 4 days after

278   inoculation compared with that in the GUS line (Fig. 3c). As previously, NRG1 expression in

279   these lines was quantified and correlated inversely with the level of leaf bacterial growth (Fig.

280   3d). These results suggest that NRG1 expression in Arabidopsis induces recognition of HopQ1

281   and quantitatively restricts *Pto* DC3000 growth in the plant.

282   **NRG1 expression is induced at the early stages of the bacterial infection**

283   As mentioned above, HopQ1 triggers an HR when expressed via *Agrobacterium* in *N.*

284   *benthamiana*. While calibrating the biolistic delivery system for transient expression in

285   Arabidopsis, HopQ1 and the GFP reporter were also delivered to *Nb* leaves as an HR

286   positive control. Surprisingly, no reduction of the GFP signal was measured when HopQ1

287   was delivered *via* biolistics in *Nb* leaves (Fig. 4a). However, when leaves were pre-treated

288   with *Agrobacterium* (not carrying any effector) to induce PTI prior to DNA bombardment,

289   a significant reduction of the GFP signal was measured. These results suggest that i)

290   unlike *Agrobacterium*-mediated transformation, the biolistic delivery of DNA bypasses

291   the PTI and maintains the cells in a non-"primed" status, and ii) NRG1 expression, and

292   consequently NRG1-dependent recognition of HopQ1, require activation of PTI for

293   efficient recognition. To confirm this hypothesis, *Nb* leaves were infiltrated with Flg22

294   peptide to induce PTI, and NRG1 expression was analysed at various times after

295   induction. NRG1 mRNA expression was strongly induced (> 5 fold) as soon as 30 minutes

296   post-induction and returned to a non-induced state after 3 h, mimicking closely the

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297   expression pattern of other well-known PTI genes, ACRE 31 and CYP71D20 (Fig. 4b). By

298   contrast a low level of NRG2 expression was detected and no induction was measured

299   upon Flg22 treatment.

Discussion

301   The HopQ1/XopQ family of effectors is highly conserved amongst plant pathogens. Recently

302   acquired in *Pseudomonas*, it is more ancient in *Xanthomonas* or *Ralstonia* and is also found

303   in *Acidovorax* (Rohmer *et al.*, 2004; Adlung & Bonas, 2017). The HopQ1 effector from

304   *Pseudomonas* and its homolog XopQ from *Xanthomonas* share a high level of sequence and

305   therefore structural similarities, suggesting a conserved yet unknown role in virulence. For

306   instance, HopQ1/XopQ share similarities to Nucleoside Hydrolases (NH) and, although no NH

307   enzymatic activity has yet been identified, the integrity of the NH domain is required for

308   virulence of *P. syringae* but dispensable for HopQ1 recognition in tobacco (Li *et al.*, 2013a).

309   HopQ1/XopQ proteins also both contain a 14-3-3 binding site, and a phosphorylation-

310   dependent interaction with 14-3-3 proteins was demonstrated for both effectors (Giska *et al.*,

311   2013; Li *et al.*, 2013b; Teper *et al.*, 2014). Again, this interaction is required for virulence but

312   not for recognition in tobacco. Finally, recognition of both effectors in *Nb* is SGT1-, EDS1- and

313   Roq1- dependent (Wei *et al.*, 2007; Adlung *et al.*, 2016; Schultink *et al.*, 2017). The multiple

314   overlaps in structure, mechanism and function of hopQ1/XopQ proteins suggests that their

315   recognition in *Nb* relies on the same detection mechanism (Adlung & Bonas, 2017). Here we

316   have demonstrated, using our previously described R gene RNAi library, that NRG1 is required

317   for the recognition of HopQ1 in *Nb* (Fig. 1) and that silencing of NRG1 promotes the

318   proliferation of *Pto* DC3000 in the plant (Fig. 2). Given its EDS1-dependency, our observation

319   that HopQ1 recognition is not solely dependent on the TNL resistance gene Roq1 but also

320   requires NRG1 which belongs to the CNL class of R proteins (Peart *et al.*, 2005) was

321   unexpected. Because of this dual dependency on both Roq1 and NRG1 we can exclude that

322   HopQ1 is recognized by two independent mechanisms involving these proteins. Since NRG1

323   was originally identified as a required component of resistance to TMV together with protein

324   N, which belongs to the TNL class, our results rather suggest that NRG1 could be involved in

325   a similar partnership with another TNL, the recently identified Roq1 protein, to mediate the

326   recognition of HopQ1. NRG1 was previously reported to belong to an atypical subclass of CC-

327   NB-LRR, named CC$_R$-NB-LRR (noted RNL hereafter) for their homology to the CC domain of

328   the RPW8 protein from Arabidopsis which confers resistance to powdery mildew (Xiao *et al.*,

329   2001). A related but separate branch of the CC$_R$ clade contains the Arabidopsis activated

330   disease resistance 1 (ADR1) protein and its homologs. NRG1 and ADR1 homologs were shown

331   to form two strongly supported subclades (Collier *et al.*, 2011). Unlike other NB-LRR proteins

332   which typically show a rapid evolutionary diversification (Jacob *et al.*, 2013), RNL members

333   display a remarkably low level of sequence variation and moderate level of clade expansion

334   (Collier *et al.*, 2011), suggesting that they perform a conserved function. Furthermore the RNL

335   clade is phylogenetically basal, and perhaps therefore of more ancient origin than the rest of

336   the CNL group. Members of the ADR1 family in Arabidopsis have a redundant function in

337   innate immunity and ETI and were suggested to act as helper NB-LRRs in partnership with

338   sensor NB-LRRs involved more specifically in effector detection (Bonardi *et al.*, 2011; Dong *et*

339   *al.*, 2016), and are also unusual among CNL proteins in requiring EDS1 (Roberts *et al.*, 2013).

340   RNL members have been reported in all plant species but, unlike ADR1 homologs which are

341   found in monocots and dicots and even gymnosperms, NRG1 homologs have been identified

342   only in dicots. Given that our evidence now shows a second occurrence of RNL-TNL

343   interaction, the co-occurrence of NRG1-like and TNL proteins in most of the dicot species and

344   their correlated absence in monocots and in some dicots such as Lamiales and *Aquilegia*

345   *coerulea* (Collier *et al.*, 2011) is of particular interest. Co-occurrence of NRG1-like and TNL

346   proteins in these species also coincides with the occurrence of the α/β hydrolase folded

347   protein Senescence-Associated Gene 101 (SAG101), which was shown to interact directly with

348   EDS1 in Arabidopsis and is typically required to trigger TNL-dependent ETI (Wagner *et al.*,

349   2013). Altogether these findings reinforce the general concept of a broader functional

350   relationship between NRG1-like and TNL proteins previously only exemplified by the N and

351   NRG1 pair but now also illustrated by the dual requirement of NRG1 and Roq1 for HopQ1

352   recognition. We note that no other hairpin constructs from the RNAi library were able to

353   cancel HopQ1-mediated HR in *Nb* and our screen thus failed to identify Roq1 as a requirement

354   for HopQ1 recognition. We have subsequently identified that the *ROQ1* gene was not

355   annotated in the *Nb* genome at the time the RNAi library was constructed and consequently

356   was not included in the final list of R genes used to build the RNAi library (Brendolise *et al.*,

357   2017).

358   We investigated whether NRG1-mediated recognition of HopQ1 could be transferred to other

359   plant species. Despite the presence of two NRG1 orthologs, AtNRG1-1 and AtNRG1-2, HopQ1

360   is not recognized in Arabidopsis. Stable transgenic Arabidopsis lines overexpressing NRG1

361   were generated and transient expression of HopQ1 in the resulting lines was shown to trigger

362   an HR. In addition, the growth of *Pto* DC3000 was also restricted when inoculated in these

363   lines (Fig. 3). These results indicate that NRG1 expression in Arabidopsis can confer HopQ1

364   recognition and confer resistance to *Pto* DC3000. These results also suggest therefore that a

365   functional ortholog of Roq1 may be present in Arabidopsis. Roq1 was reported to be highly

366   conserved in the *Nicotiana* genus but not detected in other plant species (Schultink *et al.*,

367   2017). Several potential candidates can be identified by BLASTp search of the Arabidopsis

368   annotated genome, but their low sequence homologies to Roq1 (highest was AT5G17680.1

369   with 47.1% homology) do not identify an obvious sequence ortholog despite NRG1 being able

370   to mediate the recognition of HopQ1 in Arabidopsis. This could mean that Roq1 functional

371   homologs are not restricted to the *Nicotiana* genus and might be more widely distributed in

372   plants despite poor sequence homology. The ability of NRG1 to function in Arabidopsis

373   indicates functional conservation within the NRG1 family. It would therefore be interesting to

374   investigate whether NRG1-mediated HopQ1 recognition can function in other species, in

375   particular those known to possess only the CNL type of R genes, such as monocots or Lamiales.

376   Similarly, Roq1 was reported to mediate the recognition of HopQ1 when expressed in *Beta*

377   *vulgaris* and *Nicotiana sylvestris* (Schultink *et al.*, 2017). This suggests that both species

378   contain a functional homolog of NRG1. TBLASTN search of *N. sylvestris* genome identifies two

379   sequence otholos of NbNRG1 (XP_009777016.1 and XP_009769791.1) with 96% and 76%

380   similarities respectively. However the best sequence ortholog we identify in the more distant

381   species *B. vulgaris* only shares 50% similarities with NbNRG1, yet seems to be functionally

382   able to mediate, together with Roq1, the recognition of HopQ1. These results have important

383   consequences on engineering resistance in plants by using such dual resistance components

384   as the deployment of both partners may be required to transfer the resistance to any crop of

385   interest.

386   An unusual property of NRG1 is that its expression is induced in the early stages of plant

387   infection. We show that NRG1 is at its highest level of expression as early as 30 min after

388   induction of PTI by Flg22 (Fig. 4) and returns to a non–induced state 3 h after induction of PTI,

389 suggesting that NRG1 expression is subjected to a tight regulation. That relationship between

390 mRNA expression and resistance is also evidenced by the link between transgenic expressions

391 levels of NRG1 in Arabidopsis and the ability to recognize HopQ1. The hypothesis of tight

392 regulation requirement is further reinforced by the finding that overexpression of NRG1, or

393 its CC_R domain alone, induces an HR in *Nb* (Peart *et al.*, 2005; Collier *et al.*, 2011). Interestingly,

394 although the function of HopQ1/XopQ in virulence remains unknown, several lines of

395 evidence that point towards interfering with plant innate immunity have been reported.

396 HopQ1 was reported to be one of three effectors that directly or indirectly cause the

397 degradation of RPM1-interacting protein 4 (RIN4), a negative regulator of plant innate

398 immunity in Arabidopsis and tomato, when co-expressed in *Nb* (Luo *et al.*, 2009). HopQ1 was

399 also demonstrated to inhibit innate immunity by reducing FLS2 level possibly through

400 activation of cytokinin signalling (Hann *et al.*, 2014). While several other instances of effectors

401 targeting the innate immunity have been reported, the antagonistic interplay between the

402 HopQ1 function in virulence and its detection mechanism in *Nb* would be of particular

403 interest. Since Roq1 was shown to interact physically with HopQ1 (Schultink *et al.*, 2017), we

404 propose a model where NRG1, rapidly expressed upon PTI, would together with Roq1 prime

405 the defence response (Fig. 5a). The physical interaction of HopQ1 with Roq1 would somehow

406 disrupt this balance and initiate the signalling cascade leading to cell death (Fig. 5b). It is

407 interesting to note that, similarly, the direct interaction between the TMV replicase p50 and

408 N was shown to be required to trigger the NRG1-mediated resistance to TMV (Erickson *et al.*,

409 1999; Ueda *et al.*, 2006). Whether the TNL partner (Roq1 or N) is constitutively expressed or

410 also induced by PTI remains to be determined. Any configuration where either of the two

411 partners is missing would fail to recognize HopQ1 and trigger ETI (Fig. 5c-d). Although very

412 speculative, such a model would be compatible with the SGT1-independent nature of the HR

413 triggered by overexpressing NRG1 or its CC_R domain alone (Collier *et al.*, 2011) and also

414 compatible with the SGT1- and EDS1-dependence of HopQ1 recognition due to the

415 involvement of a TNL partner. Additional analysis of protein-protein interaction between the

416 different partners will be required to support this model.

417 In conclusion, we have demonstrated that the recognition of HopQ1 in *Nb* is mediated by

418 NRG1. This result together with the recent study revealing the role of the Roq1 in HopQ1

419 recognition, is the second illustration of the concept of dual NB-LRR requirement consisting

420 of a TNL and a helper RNL to mediate effector recognition (Baggs *et al.*, 2017) and suggests

421 that NRG1 could be involved in the recognition of additional effectors through similar

422 partnerships with other TNLs. The association between the dual R protein requirement and

423 the ability of NRG1 to be induced by PTI signals provides a novel and previously undiscovered

424 degree of cooperation between PTI and ETI resistance.

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Author Contributions

434 CB and MM designed the research and wrote the manuscript together with EHAR and NP. CB

435 and RD performed the library screen with HopQ1 and subsequent identification of NRG1.

436 MMS performed stable transformation of Arabidopsis and characterization of the

437 transformants. AM and NP performed the VIGS experiments. Gene expression analysis were

438 done by MMS, MM and AM. RC and SD performed the biolistic transient expressions and

439 analysis.

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560   crinkle virus. *PLoS Pathog* **7**(11): e1002318.

Figure legends

562 Figure 1. NRG1 expression is required for HopQ1 recognition. (**a**-**b**) *Nicotiana benthamiana*

563 leaves were infiltrated by hp#26, hp#27, u135, u111 or pTKO2 empty vector (EV), and HopQ1

564 was infiltrated 48 h later. HR was monitored 4 days after HopQ1 infiltration. (**c**) Leakage of

565 electrolytes was measured on the corresponding patches as indicated. The initial time point

566 was measured two days after HopQ1 infiltration. Error bars represent the standard errors of

567 the means calculated from four biological replicates. (**d**) Schematics of the NRG1 and NRG2

568 protein structure indicating the coiled-coil (CC), nucleotide binding site, presence in APAF-1,

569 R proteins and CED-4 (NB-ARC) and leucine rich repeat (LRR) domains. Position of the u135

570 and u111 hairpin fragments are indicated (black bars). CLUSTALW alignment of u111 and u135

571 fragments with NRG1 and NRG2 nucleotide sequences. (**e**) Expression analysis of *NRG1* and

572 *NRG2* genes in u135, u111 and pTKO2 infiltrated patches relative to the *NbPP2A* gene, 4 days

573 after infiltration. Error bars represent the standard errors of the means calculated from four

574 biological replicates. Values with different letters are statistically different (p<0.01) using one-

575 way ANOVA with *post hoc* Tukey's HSD.

576 Figure 2. Silencing of NRG1 promotes *Pto* DC3000 growth in *Nicotiana benthamiana*. (**a**) Plants

577 silenced for NRG1 (TRV2-u135) or infiltrated with the corresponding empty TRV2 vector were

578 inoculated with a suspension of *Pto* DC3000 at 5×10^6 cfu/mL. Leaves were photographed 2

579 days post inoculation (DPI). See also Supporting Information Fig. S1. (**b**) *Pto* DC3000 growth was

580 evaluated immediately after inoculation and 72 h later from five leaf samples. Mean colony

581 counts harbouring a different letter are significantly different based on a paired t-test on five

582 biological replicates infiltrated by pair with the same inoculum dose (p<0.01). (**c**) *NRG1*

583   expression in the silenced plants, before *Pto* DC3000 inoculation, expressed as means ± SE

584   calculated from five leaf samples and relative to the expression of the *NbHKUK* gene.

585   Figure 3. NRG1 restricts *Pto* DC3000 growth in Arabidopsis. (**a**) Effectors HopQ1 or HopAS1,

586   or corresponding empty vector (EV), were co-expressed with a GFP reporter construct using

587   biolistics in five independent transgenic lines of Arabidopsis expressing the 35S:NRG1 or the

588   GUS control. The GFP signal was measured 24 h after DNA bombardment. Error bars

589   represent the standard errors of the means calculated from six technical replicates. One-way

590   ANOVA with *post hoc* Tukey's HSD was applied on each individual line. Values with different

591   letters are statistically different (p<0.01). See also Supporting Information Fig. S2.   (**b**)

592   Expression analysis of *NRG1* in the five 35S:NRG1 Arabidopsis transgenic lines and one of the

593   GUS lines, relative to the *AtACTIN* gene, expressed as means ± SE calculated from five leaf

594   samples. (**c**) Leaf tissues from three NRG1 lines and one GUS control were inoculated with a

595   bacterial suspension of *Pto* DC3000 at a concentration of 1×10^5 cfu/mL and bacterial growth

596   was evaluated 3 days later from six leaf samples. Error bars represent the standard errors of

597   the means and values labelled with the same letter are not statistically different based on

598   one-way ANOVA with *post hoc* Tukey's HSD (p< 0.01). (**d**) *NRG1* expression measured in the

599   same NRG1 and GUS lines evaluated in the bacterial growth assay, relative to the *AtACTIN*

600   gene and expressed as means ± SE calculated from five leaf samples.

601   Figure 4. NRG1 expression is induced by the PTI. (**a**) HopQ1 and the *GFP* reporter gene were

602   co-expressed using biolistic in *Nicotiana benthamiana* leaves pre-soaked for 10 min in an

603   *Agrobacterium* suspension OD_{600} 0.5 ([+] Agro) or in buffer ([-] Agro) and the GFP signal was

604   quantified 48 h after DNA bombardment. Error bars represent the standard errors of the

605   means calculated from four technical replicates. The experiment was repeated twice, with

606   similar results. (**b**) *N. benthamiana* leaves were infiltrated with an Flg22 solution (10 nM) to

607   induce the PTI and RNA were isolated at 0.5, 3 and 24 hours after induction. Gene expression

608   levels were quantified relative to the *NbPP2A* gene and normalised for each gene to the buffer

609   treatment at 3 hours post-induction. Error bars represent the standard errors of the means

610   calculated from four biological replicates.

611   Figure 5. Model of HopQ1 recognition. Bacteria are sensed by PRR such as FLS2 and trigger

612   PTI. NRG1 is rapidly expressed and primes the ETI response but remains in a non-activated

19

613   state through an equilibrium with its TNL partners, such as N or Roq1 (**a**). Upon injection of

614   type III effectors, Roq1 binds HopQ1 and disrupts the balance with NRG1 by a mechanism

615   which remains to be determined, and triggers ETI (**b**). In a configuration where NRG1 is not

616   present or not functional, the interaction between Roq1 and HopQ1 would occur but would

617   not be detected by NRG1 therefore no ETI would be induced and HopQ1 would progressively

618   dampen the PTI by repressing FLS2 expression. This configuration potentially corresponds to

619   Arabidopsis in which NRG1 overexpression is sufficient to mediate HopQ1 recognition (**c**).

620   Similarly in the absence of a functional Roq1, NRG1 would be induced upon PTI activation,

621   prime the ETI response through interaction with other TNLs (such as N) but in the absence of

622   Roq1, HopQ1 would not be detected by NRG1 and would eventually repress FLS2 expression.

623   This configuration would be consistent with plants species such as *Beta vulgaris* of *Nicotiana*

624   *sylvestris* in which Roq1 overexpression is sufficient to mediate HopQ1 recognition (**d**).

625 ## Supporting Information

626   Figure S1. *Pto* DC3000 promotes disease lesion in NRG1-silenced *Nicotiana benthamiana*.

627   Figure S2. HopQ1 is not recognized in Arabidopsis control lines.

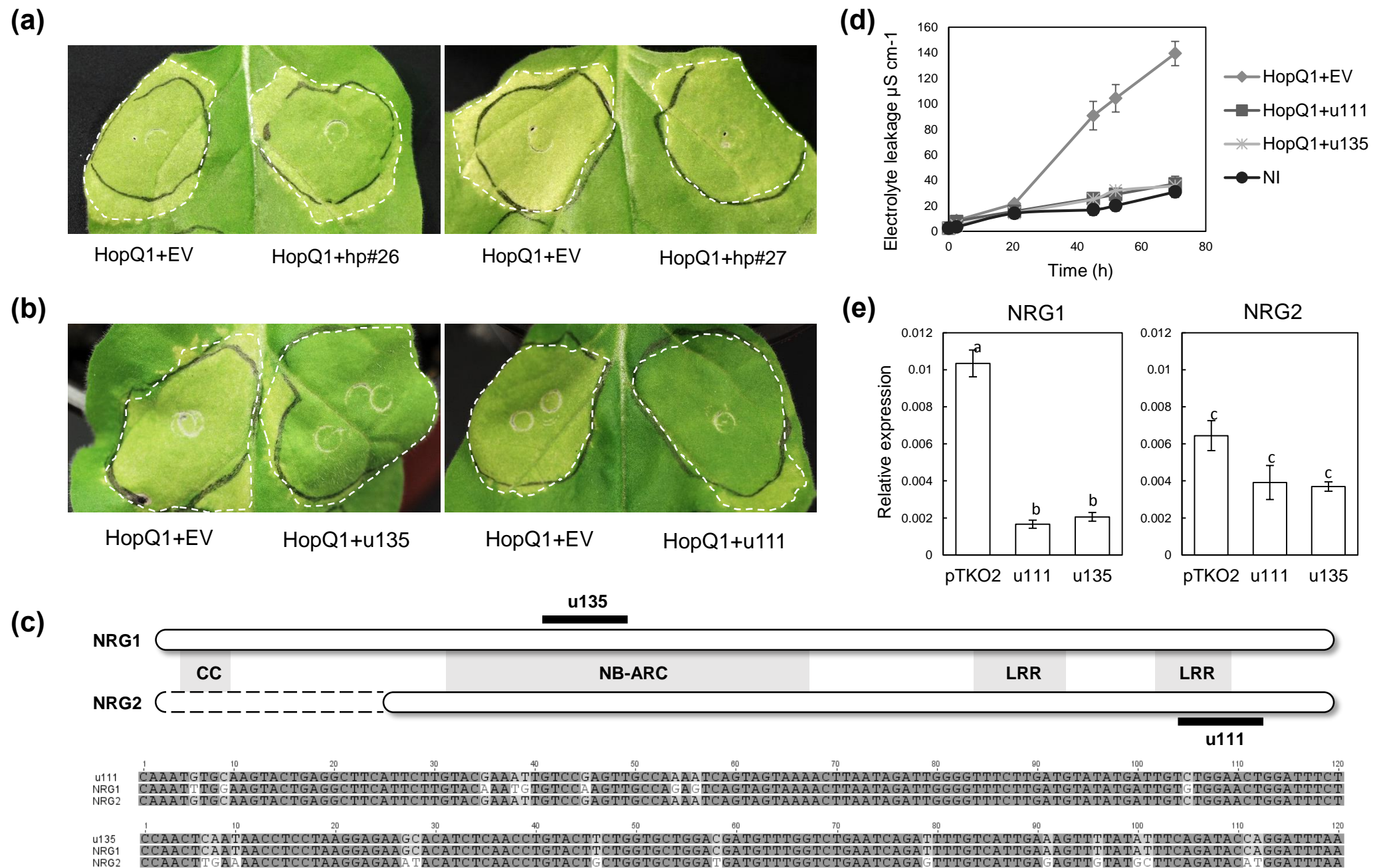628   Table S1. Sequence of the primers used in this study.

Figure 1. NRG1 expression is required for HopQ1 recognition. (**a**-**b**) *Nicotiana benthamiana* leaves were infiltrated by hp#26, hp#27, u135, u111 or pTKO2 empty vector (EV), and HopQ1 was infiltrated 48 h later. HR was monitored 4 days after HopQ1 infiltration. (**c**) Leakage of electrolytes was measured on the corresponding patches as indicated. The initial time point was measured two days after HopQ1 infiltration. Error bars represent the standard errors of the means calculated from four biological replicates. (**d**) Schematics of the NRG1 and NRG2 protein structure indicating the coiled-coil (CC), nucleotide binding site, presence in APAF-1, R proteins and CED-4 (NB-ARC) and leucine rich repeat (LRR) domains. Position of the u135 and u111 hairpin fragments are indicated (black bars). CLUSTALW alignment of u111 and u135 fragments with *NRG1* and *NRG2* nucleotide sequences. (**e**) Expression analysis of *NRG1* and *NRG2* genes in u135, u111 and pTKO2 infiltrated patches relative to the *NbPP2A* gene, 4 days after infiltration. Error bars represent the standard errors of the means calculated from four biological replicates. Values with different letters are statistically different ($p < 0.01$) using one-way ANOVA with post hoc Tukey's HSD.
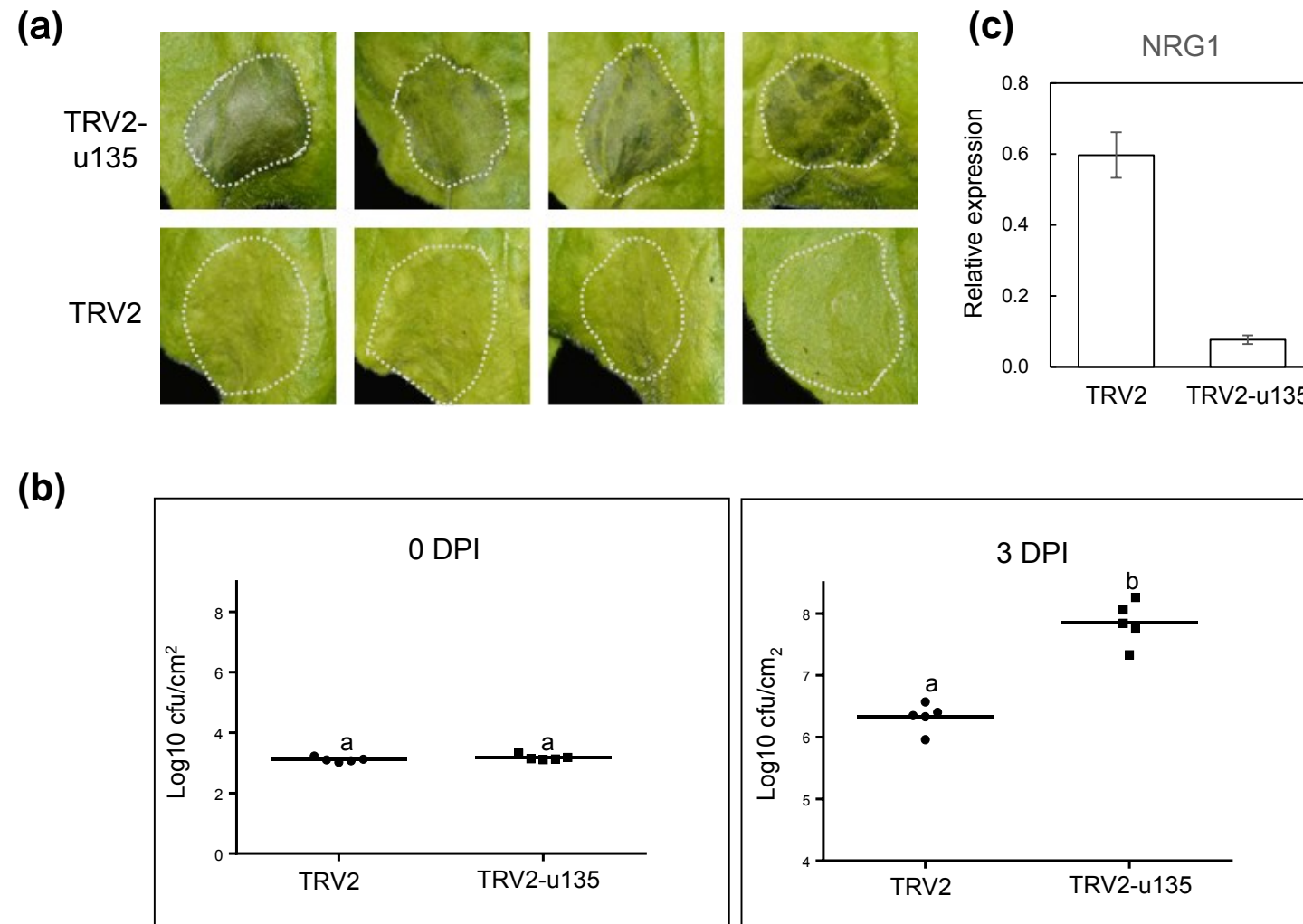
Figure 2. Silencing of NRG1 promotes Pto DC3000 growth in *Nicotiana benthamiana*. (**a**) Plants silenced for NRG1 (TRV2-u135) or infiltrated with the corresponding empty TRV2 vector were inoculated with a suspension of Pto DC3000 at 5×10^6 cfu/mL. Leaves were photographed 2 days post inoculation (DPI). See also Figure S1. (**b**) Pto DC3000 growth was evaluated immediately after inoculation and 72 h later from five leaf samples. Mean colony counts harbouring a different letter are significantly different based on a paired t-test on five biological replicates infiltrated by pair with the same inoculum dose (p<0.01). (**c**) *NRG1* expression in the silenced plants, before Pto DC3000 inoculation, expressed as means ± SE calculated from five leaf samples and relative to the expression of the *NbHKUK* gene.

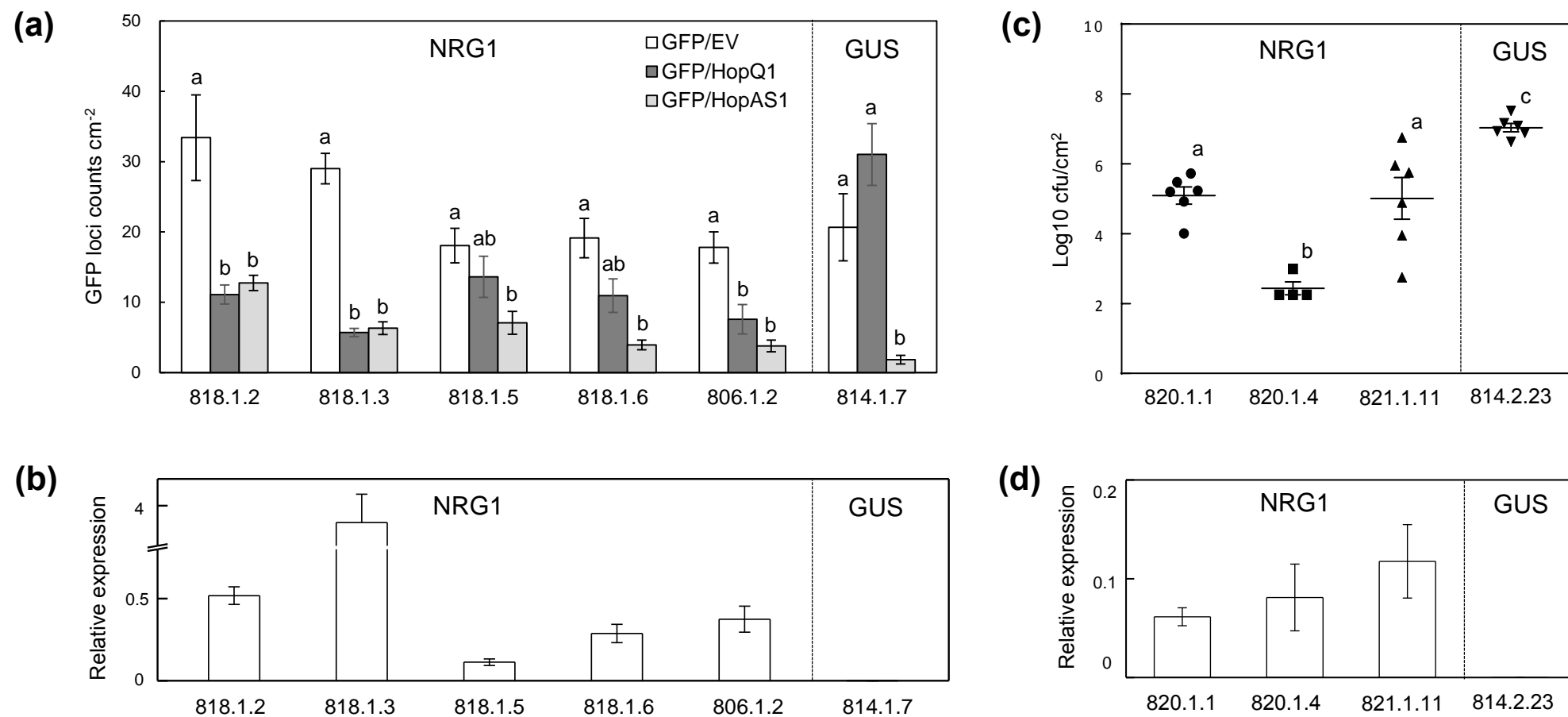Figure 3. NRG1 restricts Pto DC3000 growth in Arabidopsis. (**a**) Effectors HopQ1 or HopAS1, or corresponding empty vector (EV), were co-expressed with a GFP reporter construct using biolistics in five independent transgenic lines of Arabidopsis expressing the 35S:NRG1 or the GUS control. The GFP signal was measured 24 h after DNA bombardment. Error bars represent the standard errors of the means calculated from six technical replicates. One-way ANOVA with post hoc Tukey's HSD was applied on each individual line. Values with different letters are statistically different ($p<0.01$). See also Figure S2. (**b**) Expression analysis of *NRG1* in the five 35S:NRG1 Arabidopsis transgenic lines and one of the GUS lines, relative to the *AtACTIN* gene, expressed as means ± SE calculated from five leaf samples. (**c**) Leaf tissues from three NRG1 lines and one GUS control were inoculated with a bacterial suspension of Pto DC3000 at a concentration of 1×10^5 cfu/mL and bacterial growth was evaluated 3 days later from six leaf samples. Error bars represent the standard errors of the means and values labelled with the same letter are not statistically different based on one-way ANOVA with post hoc Tukey's HSD ($p< 0.01$). (**d**) *NRG1* expression measured in the same NRG1 and GUS lines evaluated in the bacterial growth assay, relative to the *AtACTIN* gene and expressed as means ± SE calculated from five leaf samples.
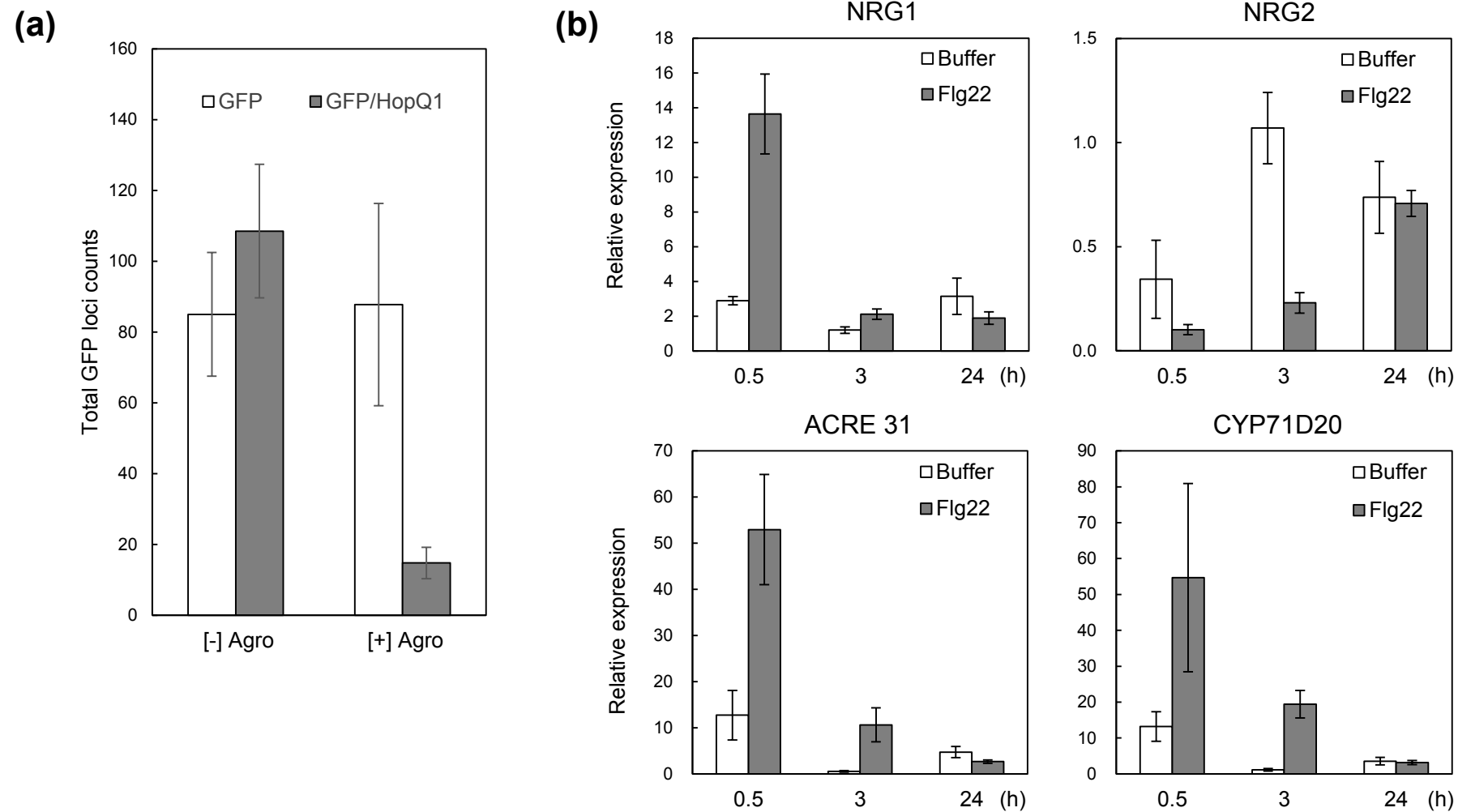
Figure 4. NRG1 expression is induced by the PTI. (**a**) *HopQ1* and the *GFP* reporter genes were co-expressed using biolistic in *Nicotiana benthamiana* leaves pre-soaked for 10 min in an *Agrobacterium* suspension OD_{600} 0.5 ([+] Agro) or in buffer ([-] Agro) and the GFP signal was quantified 48 h after DNA bombardment. Error bars represent the standard errors of the means calculated from four technical replicates. The experiment was repeated twice, with similar results. (**b**) *N. benthamiana* leaves were infiltrated with an Flg22 solution (10 nM) to induce the PTI and RNA were isolated at 0.5, 3 and 24 hours after induction. Gene expression levels were quantified relative to the *NbPP2A* gene and normalised for each gene to the buffer treatment at 3 hours post-induction. Error bars represent the standard errors of the means calculated from four biological replicates.
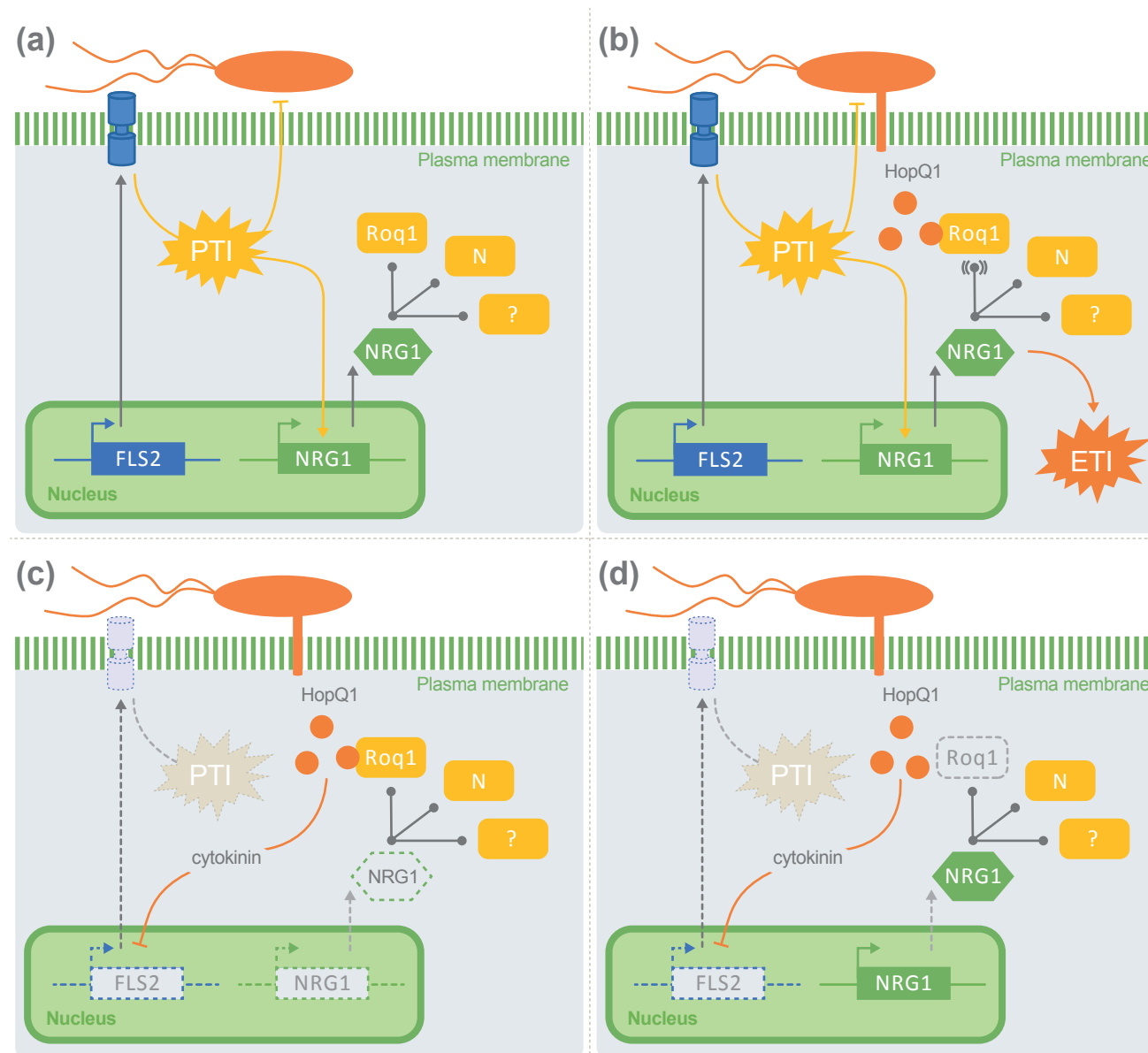
Figure 5. Model of HopQ1 recognition. Bacteria are sensed by PRR such as FLS2 and trigger PTI. NRG1 is rapidly expressed and primes the ETI response but remains in a non-activated state through an equilibrium with its TNL partners, such as N or Roq1 (**a**). Upon injection of type III effectors, Roq1 binds HopQ1 and disrupts the balance with NRG1 by a mechanism which remains to be determined, and triggers ETI (**b**). In a configuration where NRG1 is not present or not functional, the interaction between Roq1 and HopQ1 would occur but would not be detected by NRG1 therefore no ETI would be induced and HopQ1 would progressively dampen the PTI by repressing FLS2 expression. This configuration potentially corresponds to Arabidopsis in which NRG1 overexpression is sufficient to mediate HopQ1 recognition (**c**). Similarly in the absence of a functional Roq1, NRG1 would be induced upon PTI activation, prime the ETI response through interaction with other TNLs (such as N) but in the absence of Roq1, HopQ1 would not be detected by NRG1 and would eventually repress FLS2 expression. This configuration would be consistent with plants species such as *Beta vulgaris* of *Nicotiana sylvestris* in which Roq1 overexpression is sufficient to mediate HopQ1 recognition (**d**).