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# NRG1 functions downstream of EDS1 to regulate TIR-NLR-mediated plant immunity in *Nicotiana benthamiana*

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**Effector-triggered immunity (ETI) in plants involves a large family of nucleotide-binding leucine-rich repeat (NLR) immune receptors, including Toll/IL-1 receptor-NLRs (TNLs) and coiled-coil NLRs (CNLs). Although various NLR immune receptors are known, a mechanistic understanding of NLR function in ETI remains unclear. The TNL Recognition of XopQ 1 (Roq1) recognizes the effectors XopQ and HopQ1 from *Xanthomonas* and *Pseudomonas*, respectively, which activates resistance to *Xanthomonas euvesicatoria* and *Xanthomonas gardneri* in an Enhanced Disease Susceptibility 1 (EDS1)-dependent way in *Nicotiana benthamiana*. In this study, we found that the *N. benthamiana* N requirement gene 1 (NRG1), a CNL protein required for the tobacco TNL protein N-mediated resistance to tobacco mosaic virus, is also essential for immune signaling [including hypersensitive response (HR)] triggered by the TNLs Roq1 and Recognition of *Peronospora parasitica* 1 (RPP1), but not by the CNLs Bs2 and Rps2, suggesting that NRG1 may be a conserved key component in TNL signaling pathways. Besides EDS1, Roq1 and NRG1 are necessary for resistance to *Xanthomonas* and *Pseudomonas* in *N. benthamiana*. NRG1 functions downstream of Roq1 and EDS1 and physically associates with EDS1 in mediating XopQ-Roq1–triggered immunity. Moreover, RNA sequencing analysis showed that XopQ-triggered gene-expression profile changes in *N. benthamiana* were almost entirely mediated by Roq1 and EDS1 and were largely regulated by NRG1. Overall, our study demonstrates that NRG1 is a key component that acts downstream of EDS1 to mediate various TNL signaling pathways, including Roq1 and RPP1-mediated HR, resistance to *Xanthomonas* and *Pseudomonas*, and XopQ-regulated transcriptional changes in *N. benthamiana*.**

helper NLR | effector-triggered immunity | hypersensitive response | NRG1 | EDS1

Plants have evolved a sophisticated immune system to recognize and defend against virulent invading pathogens. Cell surface receptors known as pattern recognition receptors are able to perceive pathogen-associated molecular patterns (PAMPs), such as bacterial flagellin and fungal chitin, and activate innate immune response designated PAMP-triggered immunity (PTI) (1–3). However, successful pathogens employ effector molecules that can suppress PTI and lead to disease (4). In response to these effectors, plants have evolved resistance proteins (R proteins) that recognize pathogenic effectors directly or indirectly and activate a stronger and robust immune response termed effector-triggered immunity (ETI) (5–8). ETI can culminate with the development of a programmed cell death at the site of infection, also known as hypersensitive response (HR), which is correlated with pathogen growth inhibition (9).

R proteins are typically intracellular multidomain receptors of the nucleotide-binding leucine-rich repeat (NLR) type. They contain (*i*) an N-terminal coiled-coil (CC) or Toll/IL-1 receptor (TIR) domain, (*ii*) a central nucleotide binding (NB) pocket, and (*iii*) a C-terminal leucine-rich repeat (LRR) domain. Based on their N terminus, NLR proteins have been classified into CC-NLRs (CNLs) or TIR-NLRs (TNLs) (10, 11). As an example, the *Arabidopsis* TNL receptor Recognition of *Peronospora parasitica* 1 (RPP1) triggers a resistance response after binding to the effector *Arabidopsis thaliana* recognized 1 (ATR1) from *Hyaloperonospora arabidopsidis* and induces HR in *Arabidopsis* and *Nicotiana benthamiana* (12–14). Another example is the tobacco protein N, which is a TNL that binds to the helicase fragment (p50) of tobacco mosaic virus (TMV) and triggers HR and resistance to TMV (15). Although multiple plant TNLs/CNLs and their corresponding pathogenic effectors have already been defined by genetic studies, the downstream components and the molecular events involved in effector perception remain elusive. It is known that TNLs and CNLs require different signaling components to mount the ETI response. However, only a few components have been described to date. To activate immunity, most CNLs (e.g., RPM1, RPS2, RPS5) require a predicted integrin-like protein termed Non-race specific Disease Resistance 1 (16–18), whereas most TNLs (e.g., RPP2, RPP4, RPP5, RPP21, RPS4) require the lipase-like protein Enhanced Disease Susceptibility 1 (EDS1) (19–21).

## Significance

**Plants employ nucleotide-binding leucine-rich repeat (NLR) immune receptors to recognize pathogen effectors and to activate effector-triggered immunity (ETI). The Toll/IL-1 receptor-NLR (TNL) protein (Roq1) recognizes the effectors XopQ and HopQ1 in an Enhanced Disease Susceptibility 1 (EDS1)-dependent way in *Nicotiana benthamiana*. Interestingly, we found that the coiled-coil NLR protein N requirement gene 1 (NRG1) is required for activation of ETI by the TNLs Roq1 and Recognition of *Peronospora parasitica* 1. NRG1 interacts with EDS1 and acts downstream of Roq1 and EDS1 to mediate XopQ/HopQ1-triggered ETI. In addition, Roq1, EDS1, and NRG1 mediate XopQ-triggered transcriptional changes in *N. benthamiana* and regulate resistance to *Xanthomonas* and *Pseudomonas* species that carry the effectors XopQ or HopQ1. This study suggests that NRG1 may be a conserved key component in TNL-mediated signaling pathways.**

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The bacterial pathogens *Xanthomonas* and *Pseudomonas* cause severe diseases in various plants. These pathogens are Gram-negative bacteria and employ the type III secretion system (TTSS) to deliver their effector proteins into host cells. The pathogenic ability of a particular pathovar of *Xanthomonas* or *Pseudomonas* is often dependent on its specific repertoire of TTSS effectors (22, 23). Interestingly, *N. benthamiana* is resistant to the species of *Xanthomonas* and *Pseudomonas* that carry the homologous effectors XopQ and HopQ1, respectively (24, 25). We have previously shown that the TNL protein Recognition of XopQ 1 (Roq1) interacts with XopQ and HopQ1 and is required for XopQ/HopQ1-triggered HR in *N. benthamiana* (26). As for other TNL proteins, Roq1-mediated perception of XopQ is dependent on EDS1 (26–28), but the molecular mechanism for how Roq1 activation leads to ETI is largely unknown.

Recently, it has been shown that some NLR proteins function as helper NLRs for TNL- and CNL-mediated ETI signaling pathways. Examples of helper NLRs are the CNLs Activated Disease Resistance 1 (ADR1) and N requirement gene 1 (NRG1). These two NLRs are part of a subclass of CNLs whose CC domain has the closest sequence similarity to the non-NLR R protein RPW8 from *A. thaliana*. In contrast to the canonical CC domain that contains the motif "EDVID," RPW8-like CC domains do not contain this motif. In *Arabidopsis*, proteins of the ADR1 family (i.e., ADR1, ADR1-L1, and ADR1-L2) are reported to act downstream of some CNL (e.g., RPS2) and TNL (e.g., RPP2, RPP4, SNC1, CHS2) immune receptors (29, 30). On the contrary, NRG1 is reported to be required for N-mediated resistance to TMV (31). In addition to the full-length gene, a truncated version of NRG1 has also been identified in the tobacco genome but is not functional in N-mediated resistance (31). Interestingly, NRG1 is absent in some plants lacking TNLs, such as the dicot species *Aquilegia coerulea*, the dicotyledonous order Lamiales, as well as monocotyledonous species (32). Thus, it would be interesting to determine whether NRG1 serves as a common component for TNL-mediated resistance response.

In this study, we investigated the role of NRG1 in several TNL-mediated ETI pathways. We showed that NRG1 is essential for Roq1-mediated HR response and disease resistance in response to XopQ/HopQ1. In addition to N and Roq1, NRG1 is also required for the TNL protein RPP1-mediated HR triggered by ATR1 but not for the two CNL proteins Bs2- and RPS2-mediated HR, indicating that NRG1 is most likely a conserved key component in TNL-mediated immune signaling pathways. We also found that NRG1 physically associates with EDS1 and functions downstream of Roq1 and EDS1 to regulate XopQ/HopQ1-triggered ETI. Analysis of RNA sequencing (RNA-seq) data revealed that transient expression of XopQ results in substantial changes in *N. benthamiana* gene expression that are primarily mediated by Roq1, EDS1, and NRG1.

## Results

***NRG1* Is Required for Several TNL-Mediated HR Pathways in *N. benthamiana*.** To explore the molecular roles of NRG1 in ETI, especially Roq1-mediated ETI, we introduced CRISPR/Cas9-mediated mutations into the *Roq1* (*SI Appendix*, Fig. S1 *A–C*) and *NRG1* (*SI Appendix*, Fig. S1 *D–G*) genes of *N. benthamiana*. Independent frame-shift mutations including deletions and insertions were introduced into both genes, and the ETI phenotypes of the generated mutant lines including *roq1-1*, *roq1-2*, *nrg1-1*, *nrg1-2*, and *nrg1-3* were evaluated (*SI Appendix*, Fig. S1). Interestingly, keeping the infiltrated *N. benthamiana* leaves under dark conditions enhanced the effector-triggered HR; therefore, the infiltrated leaves were covered in aluminum foil during the following experiments for better observation of the HR phenotype.

We carried out *Agrobacterium*-mediated transient expression of several TNLs, CNLs, and/or their recognized effectors, including XopQ, HopQ1, RPP1+ATR1, N+p50, Bs2+AvrBs2, and Rps2 in WT *N. benthamiana*, the *roq1* and *nrg1* mutants, as well as the previously generated *eds1* mutant (26). As shown in Fig. 1*A*,

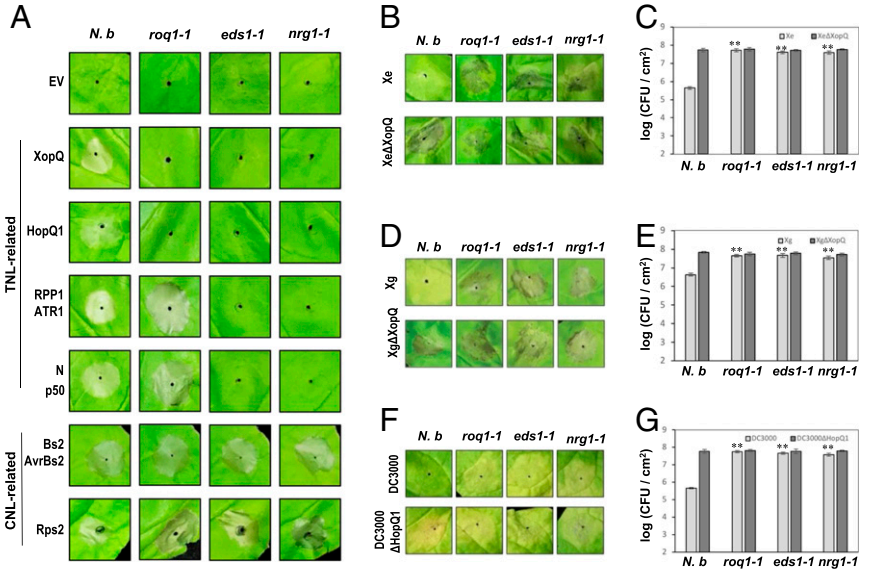


**Fig. 1.** Roles of *Roq1* and *NRG1* in effector-triggered HR and plant resistance to bacterial pathogens. (*A*) Phenotypes of HR (gray) in leaves of *N. benthamiana* (*N. b*) WT, *roq1-1*, *eds1-1*, or *nrg1-1* with *Agrobacterium*-mediated transient expression of the empty vector control (EV), XopQ, HopQ1, RPP1 plus ATR1, N plus p50, Bs2 plus AvrBs2, and Rps2. The infiltrated leaves were wrapped with aluminum foil, and images were taken 2 d postinfiltration (dpi). (*B–G*) Disease symptoms (*B*, *D*, and *F*, yellow/dark brown color) and bacterial populations (*C*, *E*, and *G*) in leaves of *N. benthamiana* WT, *roq1-1*, *eds1-1*, or *nrg1-1* after syringe infiltration with *X. euvesicatoria* (Xe) and the XopQ KO (XeΔXopQ) (*B* and *C*), *X. gardneri* (Xg) and the XopQ KO (XgΔXopQ) (*D* and *E*), or *P. syringae* pv. *tomato* DC3000 and the HopQ1 KO (DC3000ΔHopQ1) (*F* and *G*) at low inocula ($OD_{600}$ 0.0001). The disease symptoms were recorded at 12 dpi (*B*), 10 dpi (*D*), or 8 dpi (*F*), and the bacterial growth was assayed at 6 dpi (*C* and *E*) or 5 dpi (*G*). Data are means (±SD) of three biological replicates. Asterisks represent significant differences (Student's *t* test) between *N. benthamiana* and *roq1-1*, *eds1-1*, or *nrg1-1* with infiltration of *X. euvesicatoria* (*C*), *X. gardneri* (*E*), or DC3000 (*G*) (**$P < 0.01$).

transient overexpression of XopQ, HopQ1, RPP1+ATR1, N+p50, Bs2+AvrBs2, and Rps2 triggered HR in WT *N. benthamiana*. Consistent with and in support of our previous conclusion that Roq1 recognizes XopQ and HopQ1 (26), the transient overexpression of XopQ and HopQ1 failed to activate HR in the *roq1* lines, whereas RPP1+ATR1, N+p50, Bs2+AvrBs2, and Rps2 activated HR in the *roq1* mutant background (Fig. 1*A* and *SI Appendix*, Fig. S2), suggesting that Roq1 is specific for XopQ- and HopQ1-triggered ETI.

In agreement with previous findings that *EDS1* is required for TNLs-mediated ETI (27, 28), our result showed that the *eds1* mutant disrupted the HR activated by the TNL-related perception pathways for XopQ, HopQ1, N+p50, and RPP1+ATR1, but not by the CNL-related pathways for Bs2+AvrBs2 and Rps2 (Fig. 1*A*). Interestingly, we found that all *nrg1* mutants also prevented HR mediated by the TNL-related N+p50 (31), XopQ, HopQ1, and RPP1+ATR1, but not by the CNL-related Bs2+AvrBs2 or Rps2 (Fig. 1*A* and *SI Appendix*, Fig. S2), suggesting that NRG1 is probably a key component in TNL-mediated ETI signaling. Consistent results of HR phenotypes were observed in virus-induced gene-silencing plants of *NRG1* and *EDS1* (*SI Appendix*, Fig. S3). The expressions of TNLs, CNLs, and/or their recognized effectors were detected by immunoblotting (*SI Appendix*, Fig. S4), indicating that the absence of HR is not caused by a lack of protein expression.

***Roq1* and *NRG1* Are Required for Resistance to *Xanthomonas* and *Pseudomonas* in *N. benthamiana*.** Having shown that XopQ/HopQ1-triggered HR was abolished in *roq1* and *nrg1*, we then performed bacterial growth assays to examine whether *Roq1* and *NRG1* are required for resistance to *Xanthomonas* and *Pseudomonas* species and their XopQ or HopQ1 bacterial mutants.

Consistent with previous results, the XopQ KO of *Xanthomonas euvesicatoria* (XeΔXopQ) grew dramatically more than *X. euvesicatoria* and caused disease symptoms in WT *N. benthamiana* (Fig. 1 *B* and *C*) (26, 28). *X. euvesicatoria* and XeΔXopQ growth on *roq1*, *nrg1*, and *eds1* lines (26, 28) was similar to XeΔXopQ growth on WT plants. Moreover, all mutants exhibited disease symptoms (Fig. 1 *B* and *C*), indicating that Roq1 and NRG1 are required for resistance to *X. euvesicatoria*. Similar results were observed for WT and XopQ/HopQ1 KOs of *Xanthomonas gardneri* and *Pseudomonas syringae* pv. *tomato* DC3000 (Fig. 1 *D*–*G*). Based on these data (Fig. 1 *B*–*G*), we conclude that Roq1 and NRG1, together with EDS1, are collectively required for resistance to *Xanthomonas* and *Pseudomonas* in *N. benthamiana*, and function in the same ETI pathway that is triggered by XopQ/HopQ1 effectors.

**XopQ Triggers Roq1 Oligomerization in an EDS1/NRG1-Independent Manner.** We noticed that transient expression of Roq1 alone did not trigger HR in WT *N. benthamiana* and in the *roq1* line, whereas transient coexpression of XopQ and Roq1 in *roq1* did (Fig. 2*A*). As several effectors/ligands were reported to be able to trigger oligomerization of NLR receptors in plants and animals (14, 33–35), we next performed coimmunoprecipitation (co-IP) to investigate whether XopQ induces oligomerization of Roq1. As shown in Fig. 2*B*, Roq1 does not form oligomers in the absence of XopQ, whereas Roq1 oligomerization occurred when coexpressed with XopQ in *N. benthamiana* (Fig. 2*B*). Moreover, XopQ triggered Roq1 oligomerization in the *eds1* and *nrg1* lines. These results indicate that XopQ-triggered Roq1 oligomerization is an early event of ETI, and it is not dependent on EDS1 and NRG1.

**Mutation in P-Loop Motif of Roq1 Abolishes Oligomerization but Not Interaction with XopQ.** Roq1 has the typical domain structure of TNLs, with a TIR domain at its N terminus, an Apaf-1, Resistance protein, CED-4 (NB-ARC) domain (referred to as NB domain hereafter) in the middle region, and 14 putative LRRs at its C terminus (Fig. 3*A* and *SI Appendix*, Fig. S5). The NB domain contains several motifs, including the P-loop, Kin-2, RNBS-B,

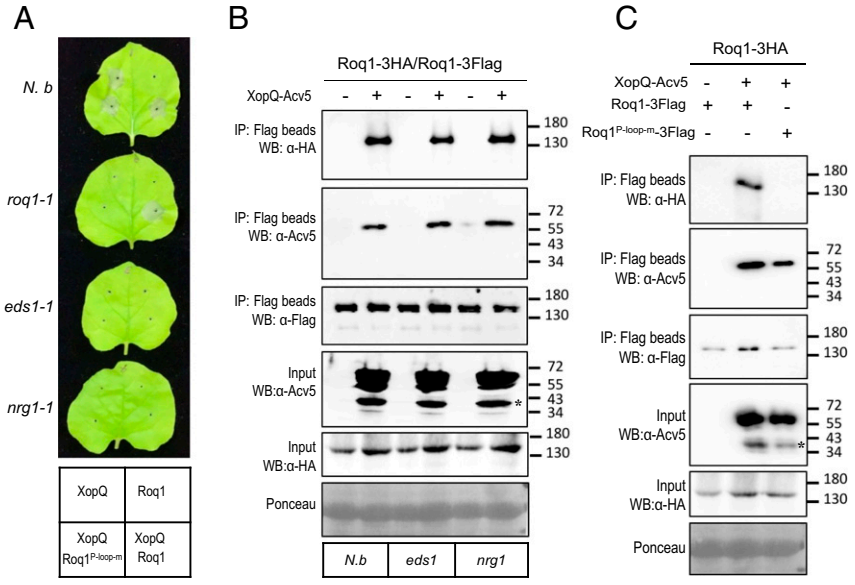


**Fig. 2.** Roq1 exhibits oligomerization in response to XopQ in an EDS1/NRG1-independent way. (*A*) Phenotypes of HR in leaves of *N. benthamiana (N. b)* WT, *roq1-1*, *eds1-1*, or *nrg1-1* with *Agrobacterium*-mediated transient expression of XopQ, Roq1, XopQ plus Roq1, or a mutated Roq1 harboring two amino acid substitutions (G223A, K224A) in the P-loop motif ($Roq1^{P\text{-}loop\text{-}m}$). Images were taken 2 dpi. (*B*) Co-IP assay showed that Roq1 displays oligomerization in response to XopQ in an EDS1/NRG1-independent way. Roq1-3HA and Roq1-3Flag were transiently coexpressed without or with XopQ-Acv5 in leaves of *N. benthamiana* WT, *eds1-1*, and *nrg1-1*. Total proteins were extracted for co-IP experiments by using α-*Flag* agarose beads and analyzed by protein gel blotting with anti-HA, anti-Flag, or anti-Acv5 antibody. Staining of RuBisCO with Ponceau S was used as a loading control. IP, immunoprecipitation; WB, Western blot. The asterisk marks the cleaved XopQ fragment. (*C*) Co-IP assay showed that the P-loop motif of Roq1 is required for its dimerization. Roq1-3HA was transiently coexpressed with Roq1-3Flag, XopQ-Acv5 plus Roq1-3Flag, or $Roq1^{P\text{-}loop\text{-}m}$ -3Flag in leaves of *N. benthamiana*. The co-IP procedure was the same as in *B*. The asterisk marks the cleaved XopQ fragment.

RNBS-C, GLPL, and MHD motifs (*SI Appendix*, Fig. S5). The P-loop motif was shown to be required for NB and essential for the function of some but not all NLR proteins (29, 33, 36–38). We next investigated whether the P-loop motif of Roq1 is required for XopQ recognition or XopQ-triggered Roq1 oligomerization.

Two key amino acids in the P-loop motif of Roq1 were substituted (G223A, K224A) to generate a mutated version of Roq1 ($Roq1^{P\text{-}loop\text{-}m}$). We found that $Roq1^{P\text{-}loop\text{-}m}$ failed to cause HR when coexpressed with XopQ in *roq1* mutant (Fig. 2*A*). Co-IP assays showed that $Roq1^{P\text{-}loop\text{-}m}$ was still able to interact with XopQ but was unable to form oligomers with Roq1 in the presence of XopQ (Fig. 2*C*), suggesting that the P-loop motif is essential for XopQ-triggered oligomerization of Roq1 and that abolishment of Roq1 oligomerization disrupts XopQ-activated HR.

**TIR of Roq1 Triggers HR in an EDS1/NRG1-Dependent Manner.** Previous studies suggested that TIR domains of some NLR proteins are responsible for NLR-mediated HR and cell death (36, 38, 39). To investigate the role of Roq1 domains, we truncated Roq1 into different fragments containing TIR, NB, and/or LRR and performed transient expression assays in *N. benthamiana* (Fig. 3*A*). We found that the short TIR fragments Roq1-TIR-A ($Roq1^{1\text{-}182}$) and Roq-TIR-B ($Roq1^{1\text{-}206}$) were unable to cause a clear HR phenotype, whereas the TIR fragments with the C-terminal–adjacent region Roq1-TIR-C ($Roq1^{1\text{-}222}$) and Roq1-TIR-D ($Roq1^{1\text{-}239}$) triggered HR in *N. benthamiana* (Fig. 3*A*), suggesting that the TIR domain of Roq1, together with the C-terminal adjacent region, is essential for triggering HR.

We further observed that overexpression of Roq1 and transient expression of its NB, LRR, or NB-LRR domains did not activate HR. However, transient expression of a truncated version of Roq1 without its NB or LRR domains (Roq1-TIR-LRR and Roq1-TIR-NB) did (Fig. 3*A*), indicating that NB and LRR domains of Roq1 may prevent TIR-triggered HR. Moreover, we noticed that Roq1-TIR-C, Roq1-TIR-D, Roq1-TIR-NB, and Roq1-TIR-LRR could not trigger HR in the *eds1* and *nrg1* lines, suggesting that the TIR domain of Roq1 activates HR in an EDS1/NRG1-dependent manner. Our co-IP assay further showed that XopQ interacts with full-length Roq1 and its LRR domain, but not with its TIR or NB domains (Fig. 3*B*), implying that the LRR domain of Roq1 is responsible for XopQ perception.

**NRG1 Exhibits Oligomerization/Dimerization and Depends on Its CC Domain to Trigger HR in *N. benthamiana*.** To investigate the function of NRG1, we truncated NRG1 into fragments containing CC, NB, and/or LRR (Fig. 4*A*), and expressed these fragments in *N. benthamiana*. As shown in Fig. 4*B*, transient expression of the CC, CC plus NB, or LRR fragments of NRG1 clearly induced HR, but the same did not happen for the fragments NB, LRR, or NB plus LRR fragments (Fig. 4*B*), consistently demonstrating that the CC domain is necessary for NRG1 in triggering HR (31, 32). We further found that NRG1-3HA physically associates with NRG1-3Flag, but not with Flag-GFP or AvrBs2-3Flag in *N. benthamiana* (Fig. 4*C*). Also, NRG1 CC-6HA associates with NRG1 CC-3Flag but not with Flag-GFP (*SI Appendix*, Fig. S6), suggesting that NRG1 and its CC domain may function as oligomers/dimers when triggering HR.

We observed that the CC-triggered HR occurred earlier in comparison with the HR caused by the fragments CC-NB or CC-LRR or by the full-length NRG1 (Fig. 4*B*), implying that NB and LRR may inhibit the CC domain function. In addition, we observed that transient expression of NRG1 driven by its endogenous promoter (NRG1pro-NRG1) did not cause HR in the WT, *nrg1*, and *eds1* lines, whereas transient expression of the NRG1 CC domain alone driven by its endogenous promoter (NRG1pro-NRG1-CC) led to a clear HR phenotype in those three lines (Fig. 4*D*). These results are consistent with the hypothesis that the NB and LRR domains of NRG1 inhibit CC-triggered HR.

The finding that the transient expression of NRG1pro-NRG1 does not cause HR in the WT, *nrg1*, and *eds1* lines also suggests that HR occurs only when a sufficient level of functional NRG1 is present. Coexpression of NRG1pro-NRG1 with XopQ and Roq1 caused HR in WT and *nrg1* lines, but not in the *eds1-1* mutant (Fig. 4*D*). By contrast, transient coexpression of XopQ/Roq1 and NRG1 carrying mutations in its P-loop (G226A, K227A) and driven by its endogenous promoter ($NRG1pro\text{-}NRG1^{P\text{-}loop\text{-}m}$) failed to cause HR in WT and *nrg1-1* lines (Fig. 4*D*), indicating that the P-loop motif of NRG1 is required for triggering HR (31).

**NRG1 Is Epistatic to Roq1 and EDS1 in Triggering HR.** Having shown that the TIR domain of Roq1 triggers HR via EDS1 and NRG1, we next investigated the genetic relations among Roq1, EDS1, and NRG1 in this process. Transient expression of XopQ or ATR1+RPP1 failed to trigger HR in *eds1-1* and *nrg1-1* mutants,

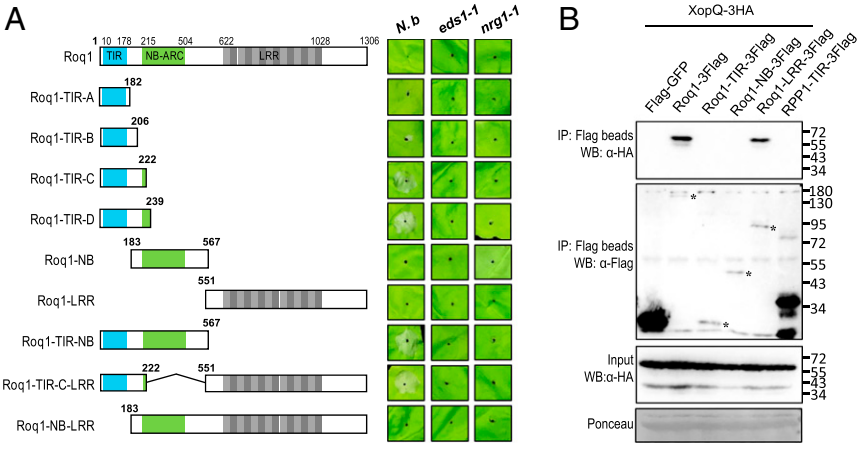


**Fig. 3.** TIR domain of Roq1 triggers HR in an NRG1/EDS1-dependent manner. (*A*) Schematic diagram (*Left*) of Roq1 domain constructs and phenotypes of HR (*Right*) in leaves of *N. benthamiana* (*N. b*) WT, *eds1-1*, or *nrg1-1* with *Agrobacterium*-mediated transient expression of the indicated domain constructs of Roq1 (*Left*). ARC is a motif present in mammal APAF-1 protein, plant R proteins, and CED4 from *Caenorhabditis elegans*. Images were taken 2 dpi. (*B*) Co-IP assay showed that XopQ interacts with LRR of Roq1. XopQ-3HA was transiently coexpressed with Flag-fused GFP, full length or domains (TIR, NB, or LRR) of Roq1, or TIR of RPP1. Total proteins were extracted for co-IP by using *α-Flag* agarose beads and analyzed by protein gel blotting with anti-Flag antibody. The asterisk marks the Flag-fused Roq1 and truncated Roq1 domain proteins. Staining of RuBisCO with Ponceau S was used as a loading control.

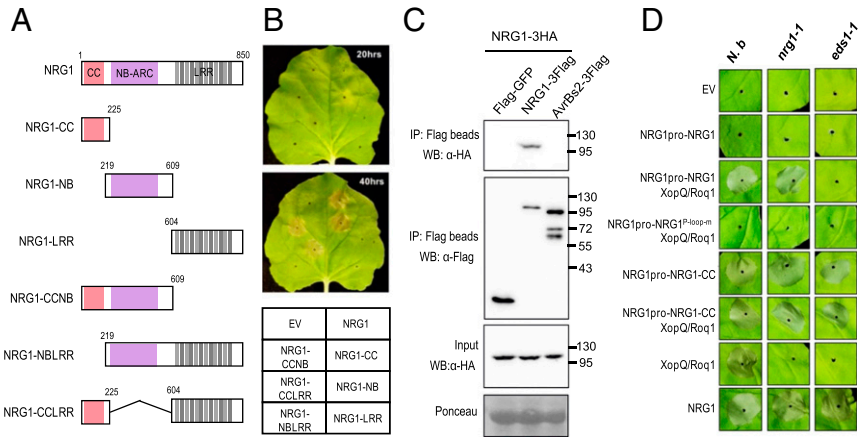


**Fig. 4.** NRG1 oligomerizes/dimerizes in *N. benthamiana* and depends on its CC domain to trigger HR. (*A*) Schematic diagram of NRG1 domain constructs with CC, NB, ARC, and/or LRR domains. (*B*) Phenotypes of HR in leaves of *N. benthamiana* (*N. b*) with *Agrobacterium*-mediated transient expression of the empty vector control (EV) and NRG1 and its domain constructs, including NRG1-CCNB, NRG1-CC, NRG1-CCLRR, NRG1-NB, NRG1-NBLRR, and NRG1-LRR. The images were taken 20 or 40 h postinfiltration. (*C*) Co-IP assay showed that NRG1 exhibits oligomerization/dimerization. NRG1-3HA was transiently coexpressed with Flag-fused NRG1, AvrBs2, or GFP in leaves of *N. benthamiana*. Total proteins were extracted for co-IP by using α-*Flag* agarose beads and analyzed by protein gel blotting with anti-HA or anti-Flag antibody. (*D*) Phenotypes of HR in leaves of *N. benthamiana* WT, *eds1-1*, or *nrg1-1* with *Agrobacterium*-mediated transient expression of the empty vector control and the indicated constructs. The endogenous *NRG1* promoter-driven NRG1 (NRG1pro-NRG1), NRG1 with two amino acid substitutions (G226A, K227A) in the P-loop motif (NRG1pro-NRG1$^{P\text{-}loop\text{-}m}$), or CC domain of NRG1 (NRG1pro-NRG1-CC) and the strong OCS promoter-driven XopQ1, Roq1, or NRG1 were transiently expressed as indicated. Images were taken 2 dpi.

whereas coexpression of EDS1 with XopQ or ATR1+RPP1 caused HR in the *eds1-1* line (Fig. 5), demonstrating that *EDS1* expression rescues the *eds1-1* phenotype. On the contrary, coexpression of EDS1 with ATR1+RPP1 or XopQ failed to trigger HR in the *nrg1 mutant* (Fig. 5), implying that *NRG1* probably acts downstream of EDS1 in ATR1+RPP1- and XopQ-triggered HR. Furthermore, transient *NRG1* overexpression caused HR in WT *N. benthamiana* and *roq1-1*, *eds1-1*, and *nrg1-1* mutants (Fig. 5), suggesting that NRG1 is epistatic to Roq1 and EDS1 in triggering HR.

**NRG1 Associates with EDS1 in *N. benthamiana*.** Having shown that NRG1 acts downstream of EDS1 to trigger HR, we further explored the relationship between NRG1 and EDS1. Our co-IP assays showed that EDS1-3HA coimmunoprecipitated with NRG1-3Flag but failed to coimmunoprecipitate with Flag-GFP, RPS2-Flag, and AvrBs2-3Flag (Fig. 6), suggesting that EDS1 associates with NRG1 in *N. benthamiana*. EDS1 was known to be able to form dimers (40) and was used as a positive control (Fig. 6*A*). We further observed that EDS1-3HA coimmunoprecipitated with NRG1-NB-3Flag and NRG1-LRR-3Flag, but not with NRG1-CC-3Flag (Fig. 6*B*), suggesting that NB and LRR domains of NRG1 may be responsible for interacting with EDS1.

**XopQ-Triggered Transcript Expression Profile Change in *N. benthamiana* Requires Roq1, EDS1, and NRG1.** To further address the roles of Roq1, EDS1, and NRG1 in the XopQ-triggered ETI pathway, we performed RNA-seq experiments by using leaves of WT, *roq1*, *eds1*, and *nrg1* lines transiently transformed with XopQ or an empty vector, which was used as a control. Three independent biological replicates were generated for each treatment, and multidimensional scaling analysis of the generated RNA-seq data showed a good correlation among the different biological replicates in each treatment (*SI Appendix*, Fig. S7).

XopQ transient expression in WT *N. benthamiana* resulted in a total of 3,070 differentially expressed transcripts [$|\log_2 FC| \geq 1$ and false discovery rate (FDR) < 0.05; Fig. 7*A*, Table 1, and *SI Appendix*, Fig. S8]. Among them, we observed repression of transcripts involved with cell cycle and division as well as with many anabolic processes, including amino acid activation and protein synthesis, photosynthesis, and nucleotide biosynthesis (Fig. 7*C*). By contrast, up-regulated transcripts were associated with biotic stress responses (e.g., PR proteins, WRKY transcription factors, 14–3-3 proteins, heat-shock proteins, peroxidases, glutathione S-transferases, calcium-binding proteins, secondary metabolite biosynthesis including phenylpropanoids, lignin, and lignans, and terpenoids) as well as with catabolic processes (e.g., carbohydrate, protein, and lipid degradation; Fig. 7 *C* and *D*). A complete list of the processes that are differentially represented in the WT and mutant lines in response to the effector XopQ is provided in *SI Appendix*, Tables S2–S4. Supporting these findings, enrichment analysis of InterPro and Gene Ontology (GO) terms also showed related domains and families that are differentially represented in response to XopQ (*SI Appendix*, Figs. S9 and S10 and Tables S5 and S6). Overrepresented InterPro terms in the set of down-regulated transcripts include Chlorophyll A-B binding protein (IPR022796), histone H2A/H2B/H3 (IPR007125), and Tubulin/FtsZ domains (IPR003008, IPR018316), whereas overrepresented terms in the set of up-regulated transcripts include WRKY domain (IPR003657), terpene synthase (IPR005630), phytocyanin domain (IPR003245), proteasome, subunit α/β (IPR001353, IPR000426), GST (IPR004046, IPR004045), and thaumatin (IPR001938). In agreement with these InterPro terms, overrepresented GO terms in the set of down-regulated transcripts include photosynthesis (GO:0015979), generation of precursor metabolites and energy (GO:0006091), and carbohydrate metabolic process (GO:0005975), whereas enriched GO terms in the set of up-regulated terms include catabolic process (GO:0009056), secondary metabolic process (GO:0019748), response to stress (GO:0006950), and response to biotic stimulus (GO:0009607; *SI Appendix*, Figs. S9 and S10).

In contrast to the large number ($N$ = 3,070) of transcripts differentially regulated in *N. benthamiana* upon XopQ expression, only 49, 2, and 785 transcripts were differentially expressed in response to XopQ in the *roq1*, *eds1*, and *nrg1* mutants, respectively ($|\log_2 FC| \geq 1$ and FDR < 0.05; Fig. 7*A* and Table 1). When comparing the XopQ transcriptional responses in the WT, *roq1*, *nrg1*, and *eds1* mutants, we observed that 3,027, 3,068, and 2,501 genes are dependent on Roq1, EDS1, and NRG1, respectively (Fig. 7*A*). These results suggest that Roq1 and EDS1 are required for most of the transcriptional changes induced by XopQ expression, and NRG1 mediates approximately

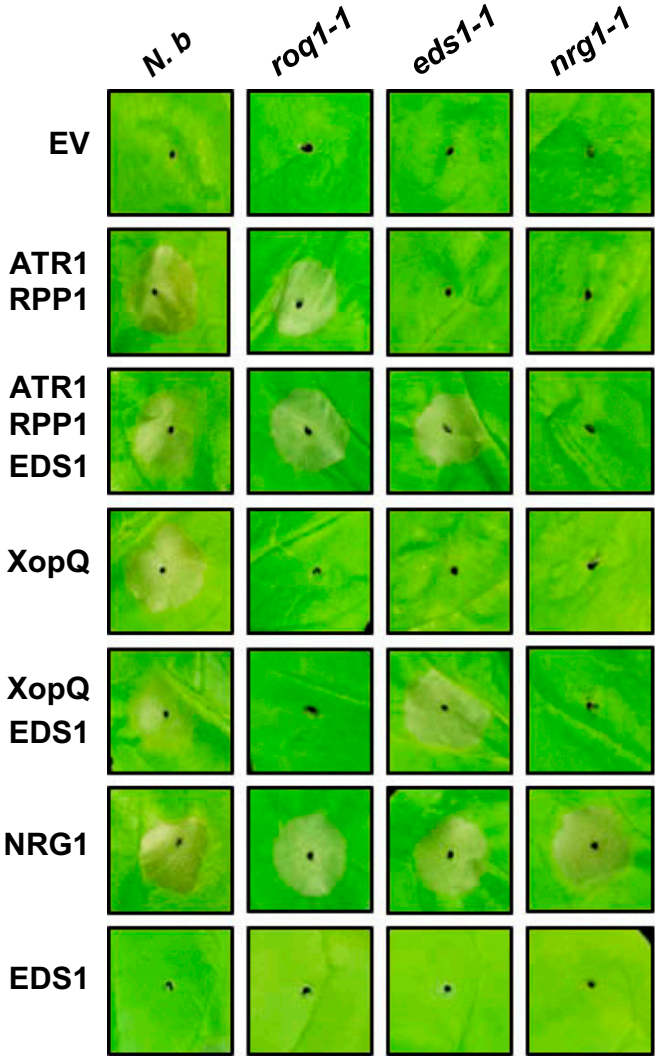


**Fig. 5.** NRG1 is epistatic to Roq1 and EDS1 in triggering HR. Phenotypes of HR in leaves of *N. benthamiana* (*N. b*) WT, *roq1-1*, *eds1-1*, or *nrg1-1* with *Agrobacterium*-mediated transient expression of the empty vector control (EV), ATR1 plus RPP1, ATR1 plus RPP1 and EDS1, XopQ, XopQ plus EDS1, NRG1, or EDS1. Images were taken at 2 dpi.

80% of these changes, with many down-regulated transcripts being independent of NRG1 (Fig. 7 *A* and *B*). We also noticed that *NRG1* expression itself is up-regulated upon XopQ expression in a Roq1/EDS1/NRG1-dependent manner, suggesting a positive feedback loop to enhance ETI. In addition, 216 transcripts are differentially expressed solely in the *nrg1* mutant in response to XopQ. Among these transcripts, we verified that the CNL ADR1 is up-regulated, thus implying a functional relationship between NRG1 and ADR1.

The expression profiles of some defense-related genes, such as *NRG1*, *ADR1*, *WRKY40*, *WRKY72*, *PR1*, and *PR5*, were confirmed by quantitative real-time (qRT) PCR. In agreement with our RNA-seq results, qRT-PCR showed that expression of *NRG1*, *WRKY40*, and *WRKY72* was clearly induced by XopQ in WT plants, whereas induction of these genes was attenuated or abolished in the *roq1*, *eds1*, and *nrg1* lines (Fig. 7*E*). Moreover, consistent with our RNA-seq results, *PR1* and *PR5* were significantly up-regulated upon XopQ expression exclusively in the WT and *nrg1* lines, whereas *ADR1* up-regulation was observed only in the absence of NRG1 in response to XopQ (Fig. 7*E*).

## Discussion

To defend themselves against pathogen attacks, plants employ NLR immune receptors that mediate the recognition of diverse pathogen effectors and trigger the ETI immune response (9, 41, 42). Interestingly, some NLR proteins function as helpers and are required for some TNL and CNL-mediated ETI signaling pathways (29–31). Elucidation of the molecular mechanisms underpinning the ETI signaling pathway will support the development of novel strategies for disease control and crop breeding.

Effectors of the XopQ/HopQ1 family are widely distributed and highly conserved among many species of the *Xanthomonas* and *Pseudomonas* genera. These effectors trigger an ETI response in *N. benthamiana*, which is resistant to *Xanthomonas* and *Pseudomonas* species (43, 44). Recently, the TNL immune receptor Roq1 was identified to mediate the recognition of XopQ/HopQ1 in *N. benthamiana*. Like other TNL proteins, Roq1 requires EDS1 to trigger an immune response (26, 28). In this study, we found that the CNL protein NRG1 is also required for XopQ/HopQ1-triggered ETI (Fig. 1). We demonstrated that Roq1, EDS1, and NRG1 are all required for resistance to *Xanthomonas* and *Pseudomonas* species (Fig. 1). In addition, transient expression of some TNLs, CNLs, and/or their related effectors in WT, *roq1*, *eds1*, and *nrg1* mutants of *N. benthamiana* showed that Roq1 is specifically associated with XopQ/HopQ1-triggered HR, whereas both EDS1 (28, 45) and NRG1 are required for several TNL-activated ETI (e.g., Roq1, RPP1, N protein), but not for CNL-mediated ETI (e.g., Bs2, Rps2; Fig. 1). These results suggest a role of NRG1 as a helper NLR in the immune signaling pathway of many TNLs. Our result is also consistent with a previous report showing that NRG1 is absent in some plants lacking TNLs, indicating an association between the occurrence of NRG1 and TNLs (32). Therefore, we suggest that, like EDS1, NRG1 might integrate signals from multiple effectors/TNLs and probably acts as a key component in the ETI activation of many TNLs (Fig.7*F*).

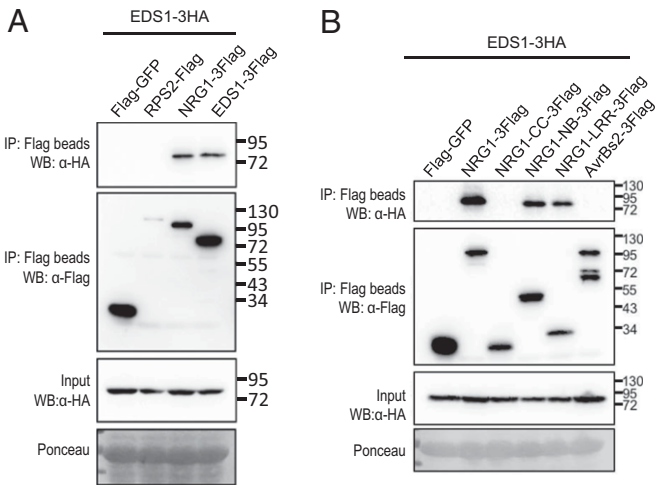


**Fig. 6.** NRG1 associates with EDS1 in *N. benthamiana* (*N. b*). (*A*) Co-IP experiment of NRG1 and EDS1. The indicated combinations of EDS1-3HA, Flag-GFP, RPS2-Flag, NRG1-3Flag, or EDS1-3Flag were transiently coexpressed in leaves of *N. benthamiana*. Total proteins were extracted for co-IP experiments by using $\alpha$-*Flag* agarose beads and analyzed by protein gel blotting with anti-HA or anti-Flag antibody. Staining of RuBisCO with Ponceau S was used as a loading control. (*B*) Co-IP experiments of NRG1 domains and EDS1. EDS1-3HA was transiently coexpressed with Flag-fused GFP, full length or domains (CC, NB, or LRR) of NRG1, or AvrBs2. Total proteins were extracted for co-IP by using $\alpha$-Flag agarose beads and analyzed by protein gel blotting with anti-Flag or anti-HA antibody. Staining of RuBisCO with Ponceau S was used as a loading control.

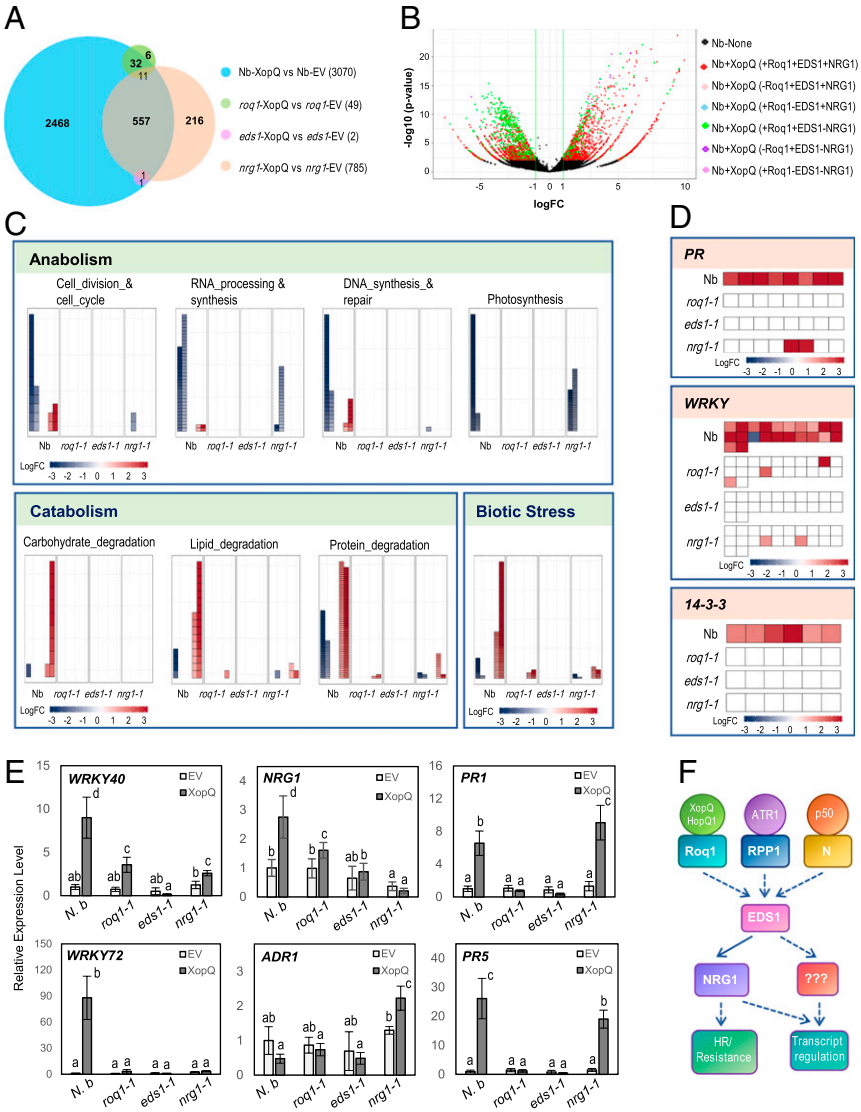


**Fig. 7.** Analysis of XopQ-initiated transcriptional changes in *N. benthamiana* (*N. b*) *roq1*, *eds1*, and *nrg1*. (*A*) The Venn diagram shows overlaps among the XopQ-regulated transcripts in WT *N. b* (Nb-XopQ vs. Nb-EV), *roq1-1* (roq1-XopQ vs. roq1-EV), *eds1-1* (eds1-XopQ vs. eds1-EV), and *nrg1-1* (nrg1-XopQ vs. nrg1-EV). The differentially regulated transcripts were selected with the criteria of $|\log_2 FC| \geq 1$ and FDR < 0.05. (*B*) Volcano plot shows the transcripts that are unresponsive to XopQ in WT *N. benthamiana* ($|\log_2 FC| < 1$ or FDR ≥ 0.05 for Nb-XopQ vs. Nb-EV) or that are responsive to XopQ in *N. benthamiana* (Nb+XopQ) and regulated in a very strict Roq1-, EDS1-, and/or NRG1-dependent manner (indicated as "+"; $|\log_2 FC| \geq 1$ and FDR < 0.05 for roq1-XopQ vs. roq1-EV, eds1-XopQ vs. eds1-EV, or nrg1-XopQ vs. nrg1-EV) or not (i.e., independent; "−"). (*C*) XopQ-triggered transcript changes involved in anabolism, catabolism, and biotic stress are attenuated in *roq1-1*, *eds1-1*, and *nrg1-1* lines. The categorization of the transcripts is based on MapMan ontology. Each box represents an individual transcript differentially regulated in response to XopQ. Up-regulated and down-regulated transcripts are shown in red and blue, respectively. The scale bar represents log fold change values. (*D*) Diagram shows expression patterns of biotic stress-related transcripts including *PR*, *WRKY*, and *14–3-3* in *N. benthamiana roq1-1*, *eds1-1*, and *nrg1-1* plants in response to XopQ. Each box represents an individual transcript. Up-regulated and down-regulated transcripts are shown in red and blue, respectively. (*E*) qRT-PCR analysis of *WRKY40*, *WRKY72*, *NRG1*, *ADR1*, *PR1*, and PR5 in WT *N. benthamiana*, *roq1-1*, *eds1-1*, and *nrg1-1* at 24 h after *Agrobacterium* infiltration with the empty vector control (EV) or XopQ. Data are means (±SD) of three biological replicates. Lowercase letters indicate significant differences by one-way ANOVA followed by least significant difference post hoc test ($P < 0.05$). (*F*) A simple model for TNL-mediated ETI signaling pathway. The receptors, including Roq1, RPP1, and N, recognize their specific effectors, including XopQ/HopQ1, ATR1, or p50, and act upstream of EDS1 and NRG1 in triggering ETI. On the contrary, uncharacterized NRG1-independent parallel branch pathway(s) may function downstream of EDS1 to regulate transcript profile change upon XopQ-triggered ETI.

Interestingly, plant NLRs show sequence similarity to animal nucleotide-binding oligomerization domain (NOD)-LRR protein family. This family of proteins includes important regulators of the inflammatory and immune responses in mammals (46, 47). The NOD domain includes a NB domain, a winged helix (WH), and helix domains (HD1 and HD2). In a closed conformation, ADP mediates the interaction between the NB domain and the WH. However, upon ligand binding to the LRR domain, conformational changes will lead to ADP exchange by ATP, self-oligomerization, and downstream signaling (47). Similarly in plants, models of NLR immune function also propose that the central NB domain functions as a molecular switch between an "on-state" (i.e., ATP-bound) and "off-state" (i.e., ADP-bound). In the presence of pathogen effectors, the off-state changes to the on-state, and, subsequently, the N terminus of the NLR oligomerizes (46, 48, 49). In agreement with this model, we observed

**Table 1. Numbers of significantly changed transcripts (|logFC| ≥ 1 and FDR < 0.05) in the indicated comparison group**

| Samples | Up-regulated | Down-regulated | Total |
|---|---|---|---|
| Nb-XopQ vs. Nb-EV | 1,485 | 1,585 | 3,070 |
| *roq1*-XopQ vs. *roq1*-EV | 48 | 1 | 49 |
| *eds1*-XopQ vs. *eds1*-EV | 1 | 1 | 2 |
| *nrg1*-XopQ vs. *nrg1*-EV | 290 | 495 | 785 |

that, upon perception of XopQ, Roq1 forms oligomers in an EDS1- and NRG1-independent manner (Fig. 2*B*). Interestingly, mutation of the P-loop motif (GxxxxGK[T/S]), which is required for NB (46), abolishes Roq1 oligomerization and XopQ-triggered HR, but not for Roq1–XopQ interaction (Fig. 2 *A* and *C*).

We also verified that the NB and LRR domains of Roq1 prevent its TIR domain from triggering HR, and the TIR domain alone can trigger HR in the absence of XopQ/HopQ1 (Fig. 3*A*). It is speculated that, in the absence of XopQ, Roq1 exists as a monomer in a conformational self-inhibition state, in which the NB and LRR domains inhibit the TIR domain. Upon XopQ perception by the LRR domain of Roq1 (Fig. 3*B*), Roq1 undergoes conformational changes and forms oligomers. Subsequently, the TIR domain is released from the NB-LRR inhibition and activates the downstream ETI pathway. The XopQ–Roq1 working model is quite similar to some other cognate elicitor–TNL models, such as ATR1–RPP1 and p50–N, as, in all these three examples, perception of effectors (XopQ, ATR1, and p50) by TNLs (Roq1, RPP1, and N) results in TNL oligomerization and activation of ETI (33, 38). An intact P-loop motif is also required for RPP1- and N-mediated HR (33, 38). Elucidation of the crystal structures of Roq1 protein and XopQ–Roq1 protein complex will help to understand the molecular mechanism by which XopQ–Roq1 initiates ETI.

Previous studies have shown that HopQ1 was cleaved when expressed in tomato and *Nicotiana* species (44, 50). Our co-IP assays showed that XopQ is also cleaved in *N. benthamiana* when its C-terminal region is fused to an Acv5 tag (Fig. 2 *B* and *C*). After cleavage, the C terminus of XopQ (Fig. 2, asterisk) is unable to interact with Roq1 (Fig. 2 *B* and *C*), implying that the mechanisms of XopQ-triggered immune defense may be more complex than we previously hypothesized. The cleaved C terminus of XopQ could not interact with Roq1, suggesting that the cleaved XopQ may lose its avirulence function, becoming unable to trigger HR. XopQ and HopQ1 were reported to interact with 14–3-3 proteins, which affect HopQ1 virulence function (44, 51, 52). It would be very interesting to elucidate whether the cleaved XopQ is still able to interact with 14–3-3 proteins, and if it still maintains its virulence function.

Coexpression of XopQ with Roq1 triggered HR in WT and *roq1 N. benthamiana*, but not in the *eds1* and *nrg1* mutants (Fig. 2*A*). NRG1 overexpression activated HR in *roq1*, *eds1*, and *nrg1* (Fig. 5), suggesting that NRG1 is epistatic to and downstream of both Roq1 and EDS1. Moreover, EDS1 overexpression with XopQ or with ATR1/RPP1 was unable to restore HR in *nrg1* (Fig. 5). These data suggest that Roq1 perceives XopQ/HopQ1 and sequentially acts through EDS1 and NRG1 to trigger ETI. Interestingly, we further found that EDS1 interacts with the NB and LRR domains of NRG1 in *N. benthamiana*, suggesting that EDS1 may affect NRG1 function through direct physical interaction. Further investigation of whether and how EDS1 affects NRG1 function will help to better understand the role of NRG1 in TNL-mediated ETI signaling pathway.

NRG1 probably exists in a self-inhibition state in plants (Fig. 4). Deletion of NB and LRR domains of NRG1 releases its CC domain, which can trigger HR even when driven by the NRG1 endogenous promoter (Fig. 4*D*). NRG1 self-inhibition might be a strategy to keep plants healthy and avoid an exacerbated defense response, thus contributing to plant survival in the natural environment. Then, in response to defense signals, NRG1, especially its CC domain, may be released to function as oligomers/dimers in the activation of plant immunity. In addition, NRG1 expression is up-regulated upon XopQ expression in an EDS1/NRG1-dependent manner (Fig. 7*E*), implying a positive feedback loop to enhance XopQ-triggered ETI.

Consistent with our genetic and biochemical analysis, our RNA-seq data further support that Roq1, EDS1, and NRG1 work together to mediate XopQ-triggered transcriptional changes. XopQ expression activates a considerable transcriptional reprogramming in the plant tissues. Transcripts related to plant biotic stress responses, such as PR proteins, WRKY transcription factors, secondary metabolite biosynthesis, and oxidative stress, are up-regulated in response to XopQ (Fig. 7 *C* and *D* and *SI Appendix*, Fig. S11), indicating that plant ETI response is activated. However, defense activation seems to be triggered to the detriment of other cellular processes, such as cell cycle and division as well as biosynthesis of macromolecules (i.e., proteins and nucleotides; Fig. 7*C*). Also, many catabolic processes, such as carbohydrate, protein, and lipid degradation, seem to be activated in response to the effector (Fig. 7*C*), suggesting that the plant is redirecting its energy to mount a defense response triggered by XopQ. Remarkably, these transcriptional changes are clearly attenuated in the *roq1*, *eds1*, and *nrg1* mutants (Fig. 7). Roq1 and EDS1 are responsible for most transcriptional changes, whereas NRG1 is required for only approximately 80% of these changes, suggesting that other protein(s) might function downstream of EDS1 and probably work in parallel with or to compensate for the absence of NRG1 in the mediation of XopQ-triggered transcript expression changes (Fig. 7*F*).

We also observed that the defense gene PR1, which is induced in response to a variety of pathogens (53, 54), is still up-regulated in the *nrg1* mutant upon XopQ expression, indicating that NRG1 is not required for all defense-related transcriptional changes that occur in response to XopQ. Another interesting result is that proteins from the 14–3-3 family, which are up-regulated in the WT line upon XopQ expression, are no longer differentially expressed in the *nrg1* mutant (Fig. 7*D*). Increasing evidence supports a role of these proteins in signal transduction during plant immune responses at diverse levels (55, 56), and our data suggest that NRG1 is required for their up-regulation during plant immune responses. Another interesting finding is that the helper NLR ADR1, which, along with NRG1, is part of the RPW8-like CC domain subclass, is up-regulated only in the *nrg1* mutant upon XopQ expression (Fig. 7*E*). Given that hundreds of transcripts are still differentially regulated in the *nrg1* mutant in response to XopQ, it would be interesting to know if these transcripts are regulated by another helper NLR, such as ADR1. Future experiments to understand additional details about NRG1 function in plant immunity and its conformational changes during ETI will shed light on the molecular mechanisms/events underpinning TNL-mediated ETI signaling pathway.

## Materials and Methods

Details of the materials and methods used in this paper, including generation of *N. benthamiana* mutants and plant growth conditions, virus-induced gene silencing, HR phenotype, in planta bacterial-growth assays, qRT-PCR analysis, co-IP assay, RNA-seq, and gene expression analysis, are provided in *SI Appendix, Supplementary Information Text: Materials and Methods*.

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