

Two genetically linked *Arabidopsis* TIR-type NLRs are required for immunity and interact with NLRs encoded in a segmentally duplicated genomic region

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Abstract

Plant nucleotide-binding leucine-rich repeat (NLR) immune receptors detect pathogen-secreted effectors inside host cells and induce a robust immune response, typically involving hypersensitive cell death. NLR genes are often genetically linked and can function as pairs or within larger NLR networks. We previously showed that the truncated Toll/Interleukin-1 Receptor (TIR)-type NLR TIR-NB13 (*TN13*) is required for resistance of *Arabidopsis* to *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 lacking the type-III effector proteins AvrPto and AvrPtoB. *TN13* is genetically linked to a full length *TIR-NB-LRR* (*TNL*) gene on chromosome 3. Here, we show that *TN13*, and its genetically linked *TNL* both localize to the ER membrane via N-terminal transmembrane domains, are required for resistance to *Pst* DC3000 (Δ AvrPto/AvrPtoB) and interact with each other in transient expression assays in *Nicotiana benthamiana*. In contrast to *TN13*, the full length *TNL*, which we named *TN13-INTERACTING TNL1* (*TNT1*), induces an autoactive cell death response when expressed in *N. benthamiana* that depends on an atypical MHV motif in its NB-ARC domain, as well as the EDS1/SAG101/NRG1 module. *TN13* and *TNT1* furthermore interact with phylogenetically related NLRs encoded by a segmentally duplicated region on chromosome 5. Our data suggest that both *TN13* and the genetically linked *TNT1* could be part of a larger TIR-type NLR immune regulatory network, in which *TNT1* contributes to basal immunity and might function as an autoactive death switch to induce cell death upon pathogen detection.

SIGNIFICANCE STATEMENT

The ER membrane localized truncated TIR-NLR *TN13* and the genomically linked full length *TNL* *TNT1* are required for plant disease resistance and form heteromeric associations with phylogenetically related NLRs, encoded by a segmentally duplicated chromosomal region.

INTRODUCTION

Plants evolved a multilayered, cell autonomous immune system to efficiently detect and respond to pathogen threats (Dangl *et al.*, 2013). Nucleotide-binding and leucine-rich repeat (NLR) proteins function as intracellular immune receptors to sense pathogen-secreted effector molecules that contribute to pathogen virulence, if they remain undetected (Kourelis and van der Hoorn, 2018; Wu and Derevnina, 2023). Effector recognition by NLRs initiates a robust defense response often including a locally restricted programmed cell death, known as the hypersensitive response (HR), to restrict the growth of biotrophic pathogens that require living host tissues (Dangl *et al.*, 2013).

NLRs have a modular, multi-domain architecture with a central NB domain that contains Apaf-1/R-protein/CED4 (ARC1/2) subunits (NB-ARC), a C-terminal LRR-domain and either a Toll/Interleukin-1 receptor (TIR) domain or a coiled-coil (CC) domain at the N-terminus. Thus, NLRs can phylogenetically be divided into distinct classes, referred to as TIR-type NLRs (TNLs) and CC-type NLRs (CNLs; Shao *et al.*, 2016; Kourelis *et al.*, 2021; Contreras *et al.*, 2023).

Plant NLRs belong to the family of signal transduction ATPases with numerous domains (STAND) and are proposed to function as ATP-hydrolyzing molecular switches (Leipe *et al.*, 2004; Takken *et al.*, 2006; Danot *et al.*, 2009). In the absence of a cognate effector molecule or an activating trigger, NLRs are in a resting state and bind ADP with their NB-ARC domain (Williams *et al.*, 2011; Wang, Wang, *et al.*, 2019; Selvaraj *et al.*, 2024). An effector triggered switch to the active state requires the substitution of ADP by ATP (Zhou *et al.*, 2015; Wang, Hu, *et al.*, 2019; Selvaraj *et al.*, 2024). The ADP-ATP exchange and the stabilization of ATP relies on a functional phosphate-binding loop (P-loop) within the NB domain (Wang, Hu, *et al.*, 2019). In addition to the P-loop, the histidine of the conserved MHD (Met-His-Asp) motif within the ARC2 domain also interacts with the ADP/ATP β -phosphate (Reubold *et al.*, 2011). An amino acid substitution from MHD to MHV can render plant NLRs autoactive, presumably by shifting the binding preference from ADP towards ATP (Bendahmane *et al.*, 2002; Tameling *et al.*, 2006; Williams *et al.*, 2011). The nucleotide-bound state also correlates with intra-molecular interactions between different NLR domains (Ve *et al.*, 2015; Bentham *et al.*, 2018). The LRR domain directly interacts with parts of the NB-ARC domain to maintain the NLR in a repressed state (Slootweg *et al.*, 2013; Schreiber *et al.*, 2016). Direct interactions with effectors or sensing of effector-induced host protein modifications by LRR domains can release intra-molecular auto-inhibitory effects for ATP binding and NLR activation (Slootweg *et al.*, 2013; Ntoukakis *et al.*, 2013).

Plant NLRs rarely function as singletons, which are capable of both effector detection and cell death induction, but rather form NLR pairs or complex networks which separates effector detection and downstream signaling and immunity (Lüdke *et al.*, 2022; Contreras *et al.*, 2023). Accordingly, sensor NLRs perceive effectors, and signal through helper NLRs to initiate immune signaling (Qi *et al.*, 2018; Wu *et al.*, 2017; Castel *et al.*, 2019; Lüdke *et al.*, 2022; Contreras *et al.*, 2023). All currently identified full length TIR-type NLRs act as sensors that either signal through ADR1 (ACTIVATED DISEASE RESISTANCE1) or NRG1 (N REQUIREMENT GENE1) as helper NLRs for induction of immunity or HR (Wu *et al.*, 2019; Lapin *et al.*, 2019; Saile *et al.*, 2020). Inhibitory inter-molecular interactions between sensor NLR pairs are an additional regulatory layer to prevent signaling in the absence of a pathogen (Césari *et al.*, 2014; Huh *et al.*, 2017). For instance, effector perception by the inhibitory *Arabidopsis* TNL sensor RESISTANCE TO RALSTONIA SOLANACEARUM1 (RRS1-

R) leads to dissociation from the TNL partner RESISTANCE TO PSEUDOMONAS SYRINGAE4 (RPS4), which acts as an executor to induce an immune response that depends on the helper NLR NRG1 (Sarris *et al.*, 2015; Le Roux *et al.*, 2016; Huh *et al.*, 2017; Ma *et al.*, 2018; Castel *et al.*, 2019). The helper NLR classes of NRG1s and ADR1s form a subgroup of NLRs, in which the N-terminal CC domain resembles the CC motif of the resistance protein RESISTANCE TO POWDERY MILDEW8 (RPW8; Peart *et al.*, 2005; Bonardi *et al.*, 2011; Dong *et al.*, 2016). While some *Arabidopsis* NLRs require both ADR1 and NRG1, others require either NRG1 or ADR1 for immune signaling (Castel *et al.*, 2019; Wu *et al.*, 2019). Besides helper NLRs, the defense regulator ENHANCED DISEASE SUSEPTIBILITY1 (EDS1) is essential for TIR-type NLR mediated immunity and is also required for signaling of some CNLs (Wiermer *et al.*, 2005; Xiao *et al.*, 2005; Wagner *et al.*, 2013). The EDS1-related proteins PHYTOALEXIN DEFICIENT4 (PAD4) and SENESCENCE ASSOCIATED GENE101 (SAG101) function together with EDS1 and form distinct hetero complexes (Feys *et al.*, 2005; Huang *et al.*, 2022; Feehan *et al.*, 2023). Whereas signaling via an EDS1-PAD4-ADR1 module restricts bacterial growth through transcriptional reinforcement, EDS1-SAG101-NRG1 were proposed to signal mainly for HR induction (Bonardi *et al.*, 2011; Dong *et al.*, 2016; Cui *et al.*, 2018; Castel *et al.*, 2019; Lapin *et al.*, 2019; Saile *et al.*, 2020; Feehan *et al.*, 2023). Following effector detection by TIR-type NLR sensors, NRG1 and ADR1 helper NLRs oligomerize and localize to the plasma membrane where they mediate Ca²⁺ influx to induce immune signaling (Jacob *et al.*, 2021; Feehan *et al.*, 2023). Activated NRG1 was also shown to target other organellar membranes, such as the chloroplast (Ibrahim *et al.*, 2024).

TIR-type NLR sensors function as NAD⁺/NADP⁺ degrading enzymes and produce cyclic ADP-ribose variants (v-cADPR) that are required for cell death induction (Horsefield *et al.*, 2019; Jia *et al.*, 2022). While ADP-ribosylated ATP (ADPr-ATP) and ADPr-ADPR (di-ADPR) binding to EDS1-SAG101 dimers promotes NRG1 association and oligomerization (Jia *et al.*, 2022), 2'-(5'-phosphoribosyl)-5'-adenosine mono-/diphosphate (pRib-AMP and pRib-ADP) bind to EDS1-PAD4 dimers leading to ADR1 association and oligomerization (Huang *et al.*, 2022). In addition, it was recently shown that the 2',3'-cAMP/cGMP synthetase activity of TIR domain proteins such as the TIR-only NLR RESPONSE TO THE BACTERIAL TYPE III EFFECTOR PROTEIN HOPBA1 (RBA1) also contributes to cell death induction (Yu *et al.*, 2022). RBA1 is required for immunity to *Pseudomonas syringae* expressing the effector protein HopBA1, and induces an HR cell death response when expressed transiently in *Nicotiana benthamiana* (Nishimura *et al.*, 2017). Other truncated NLRs encoded by the *Arabidopsis* genome include TIR-NB (TN) and TIR-X (TX) proteins that lack NB and/or LRR domains but might contain additional (X) domains (Meyers *et al.*, 2002; Nandety *et al.*, 2013; Van de Weyer *et al.*, 2019). A functional role of truncated NLRs in immunity has been described for TN2, which is required for defense signaling in the *exo70b1-3* auto-immune mutant, suggesting that TN2 monitors the integrity of EXOCYST SUBUNIT EXO70 FAMILY PROTEIN B1 (EXO70B1), involved in vesicle exocytosis (Zhao *et al.*, 2015). In addition, TN2 monitors the cellular homeostasis of the E3 ligase SENESCENCE-ASSOCIATED E3 UBIQUITIN LIGASE1 (SAUL1) together with the TN-type NLR CHILLING SENSITIVE1 (CHS1/TN1), both of which depend on the TNL SUPPRESSOR OF CHS1 (SOC3; Zhang *et al.*, 2017; Tong *et al.*, 2017; Liang *et al.*, 2019). Significantly, SOC3 forms a genomic cluster with CHS1 and TN2, a chromosomal arrangement commonly observed among NLRs that function cooperatively (Meyers *et al.*, 2003; van Wersch and Li, 2019).

We previously showed that the ER-localized truncated NLR TIR-NB13 (TN13) is required for immunity of *Arabidopsis* to mildly virulent *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (Roth *et al.*, 2017). The gene encoding TN13 (AT3G04210) is linked genetically in a head-to-tail orientation to a predicted full length *TNL* gene (AT3G04220).

Here, we report that AT3G04220, which we named TN13-INTERACTING TNL1 (TNT1), interacts and co-localizes with TN13 at the ER membrane via its N-terminal transmembrane domain, and is also required for immunity to mildly virulent *Pst* DC3000. Interestingly, TNT1 contains an atypical MHV motif in its wild-type amino acid sequence, which is associated with autoactivity and appears not to be present in other *Arabidopsis* NLRs. We show that this motif is required for the induction of HR when expressed transiently in *N. benthamiana*. The cell death response further depends on a predicted NAD⁺/NADP⁺ degrading catalytic glutamate residue present in the TIR domain, and the *NbEDS1a/NbSAG101b/NbNRG1*, but not on the *NbPAD4/NbADR1* module. The genomic region containing *TN13* and *TNT1* on chromosome 3 was segmentally duplicated in the *Arabidopsis* genome and contains an expanded number of *TNL* genes on chromosome 5. TN13 and TNT1 interact with each other and with phylogenetically closely related NLRs of the duplicated region, suggesting the potential formation of a functional NLR network regulating plant immunity. We propose that TNT1 acts as an autoactive death switch that is regulated by interactions with TN/TNL proteins. Effector detection could activate immune signaling and cell death induction by TNT1 at the cytoplasmic side of the ER membrane.

RESULTS

***TN13* and *TNT1* mutants show enhanced susceptibility to *Pst* DC3000 (Δ AvrPto/AvrPtoB)**

We previously identified the truncated NLR protein TIR-NB13 (TN13) as a component of immunity against mildly virulent *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 lacking the type-III effector proteins AvrPto/PtoB (Roth *et al.*, 2017). On *Arabidopsis* chromosome 3, *TN13* is genetically linked in a head-to-tail orientation to a predicted full length *TNL* gene (AT3G04220, Figure 1A) which we named *TN13-INTERACTING TNL1* (*TNT1*). We hypothesized that the genetic linkage suggests cooperative functions in immunity (van Wersch and Li, 2019). We therefore investigated the requirement of *TNT1* in immunity by inoculating homozygous T-DNA insertion mutants with *Pst* DC3000 (Δ AvrPto/AvrPtoB) (Figures 1B, S1). The *tnt1* mutant line showed enhanced susceptibility towards *Pst* DC3000 (Δ AvrPto/AvrPtoB) to an extent that is comparable to the *tn13* mutant line (Figure 1). Consistent with cooperative functions in plant defense against *Pst* DC3000, a *tn13 tnt1* deletion mutant that was generated by CRISPR/Cas9 genome editing, shows susceptibility that is comparable to the *tn13* and *tnt1* single mutants (Figure 1B, S1). The Col *eds1-2* mutant was used as a hyper-susceptible control, whereas the auto-immune mutant *snc1* (*suppressor of npr1-1, constitutive 1*) served as a control for enhanced resistance (Figure 1B). The enhanced susceptibility of the *tn13* and *tnt1* mutant lines was complemented by stable expression of the respective wild-type proteins with a C-terminal mYFP tag, under control of either the native (*Np*) or the constitutively active 35S promoter, respectively (Figure S2). Stable transgenic expression of TN13-mYFP by the 35S promoter resulted in a stunted phenotype commonly associated with activated immunity (Figure S2A). The *tn13* and *tnt1* mutant lines and the *tn13 tnt1* double mutant appeared wild-type-like and did not display signs of autoimmunity

(Figure 1, S2A). We conclude that the truncated TIR-type NLR *TN13*, and the genomically linked full length NLR *TNT1*, are both required for immunity against mildly virulent *Pseudomonas* bacteria.

TNT1 induced cell death depends on *EDS1*, *SAG101b* and *NRG1* in *Nicotiana benthamiana*

Based on domain predictions, TNT1 contains canonical TIR-type NLR domains (TIR, NB-ARC and LRR; Figure S3). As observed for TN13 (Roth *et al.*, 2017), TNT1 also contains a predicted N-terminal hydrophobic transmembrane (TM) domain, which is predicted to have an alpha helical structure (Figures S3B). We previously reported that TN13 localizes to membranes of the endoplasmic reticulum (ER) and the nuclear envelope after transient expression in *N. benthamiana* (Roth *et al.*, 2017). To investigate the subcellular localization of TNT1, we performed transient expression assays of mYFP-tagged TNT1 in *N. benthamiana*. Two days after *Agrobacterium*-infiltration for expression of 35S promoter or *Np*-driven TNT1-mYFP, we noticed a cell death response, resulting in tissue collapse five days after infiltration (Figure 2A). Cell death was also observed upon transient expression of a C-terminal 3xHA-StrepII-tagged version of TNT1, whereas transient expression of TN13-mYFP did not induce cell death (Figures 2A, S4). To further investigate whether the cell death response depends on EDS1, as previously reported for TIR-type NLRs, we transiently expressed TNT1-mYFP in *Nbeds1a-1* mutant plants (Ordon *et al.*, 2017). No cell death could be observed five days after *Agrobacterium*-infiltration, although the full-length fusion protein accumulated (Figures 2B, S4). We utilized *N. benthamiana* single, double and triple mutant combinations of the EDS1 interaction partners PAD4 and SAG101, and the helper NLRs *NRG1* and *ADR1* to elucidate their requirements for cell death induction upon TNT1-mYFP expression. Since an *adr1* mutant was not available at the time, we complemented the *adr1 nrg1* double mutant by co-expressing an untagged version of *NbNRG1* (Lapin *et al.*, 2019). HR cell death upon TNT1-mYFP expression fully depends on *NbSAG101b* and *NbNRG1*, whereas *NbSAG101a*, *NbPAD4* and *NbADR1* are dispensable (Figures 2B, S4). The transient expression assays indicate that TNT1 autoactively signals for cell death through the EDS1-SAG101-NRG1 module in *N. benthamiana*.

TNT1 co-localizes with the genomically linked TN13 to membranes of the ER and the nuclear envelope

For subcellular localization studies, we transiently co-expressed TNT1-mYFP with a CFP-tagged ER marker (ER-ck; Nelson *et al.*, 2007) in *Nbeds1a-1* mutant plants. Confocal laser scanning microscopy (CLSM) revealed that TNT1-mYFP, like TN13-mYFP, co-localizes with the ER-ck marker (Figure 3). The same localization was observed when expressing TNT1 or TN13 transiently under control of the respective *Np* (Figure S5). We analyze the subcellular localization of TN13-mYFP and TNT1-mYFP in *Arabidopsis* by using stable transgenic complementation lines expressing the respective mYFP fusion constructs under control of the *Np* or 35S promoter (Figure S1). Fluorescence signals could not be detected for lines expressing the fusion protein constructs under control of the respective *Np*. For lines expressing TN13-mYFP or TNT1-mYFP under control of the 35S promoter, we detected a fluorescence signal at the nuclear periphery and the ER (Figure S6). This is consistent with the observed localization in *N. benthamiana* and with the predicted N-terminal TM domains for TN13 and TNT1 (Figures 3, S3). Our data suggest that both TN13 and the genetically linked TNT1 localize to the ER membrane via their N-terminal TM domains.

TNT1-induced cell death depends on an atypical MHV motif

To investigate whether TNT1-triggered cell death in *N. benthamiana* depends on previously described functional NLR motifs, we conducted site-directed mutagenesis of the phosphate-binding loop (P-loop) and the MHD motif within the NB-ARC domain (Figure 4A). We found that the wild-type TNT1 protein contains an atypical MHV motif (Figures 4B). Mutations of MHD motifs to MHV have previously been shown to render NLRs auto-active (Bendahmane *et al.*, 2002; Tameling *et al.*, 2006; Williams *et al.*, 2011). We surveyed a NLR dataset from 180 RefSeq reference plant proteomes (Toghiani and Kamoun, 2024) and extracted MHD motifs of 56,686 NLRs to analyze the amino acid frequencies within the MHD motif. As expected, MHD constitutes the consensus sequence, and only a low number of NLRs (0.226 %) naturally contain a Valin (V) in the 3rd position of the MHD motif (Figure 4B). In *Arabidopsis*, TNT1 is the only NLR containing an MHV motif (Supplementary Dataset 2). Mutating the wild-type MHV-motif of TNT1 to MHD or MHH abolished the cell death response upon transient expression in *N. benthamiana* wild-type plants, albeit protein accumulation was comparable to the TNT1 wild-type protein expressed in *Nbeds1a-1* (Figures 4, S7A). Mutagenesis of the P-loop in the wild-type TNT1 protein (containing the MHV motif) also abolished the cell death induction in wild-type *N. benthamiana* (Figures 4, S7B). We conclude that TNT1 requires the atypical MHV motif for the autoactive induction of cell death in a P-loop dependent manner.

The TIR-domain of TNT1 is sufficient to trigger cell death in *N. benthamiana*

Expression of TIR domains was shown to be sufficient for cell death induction in *N. benthamiana* (Bernoux *et al.*, 2011; Nandety *et al.*, 2013; Williams *et al.*, 2014; Nishimura *et al.*, 2017). To analyze if the TNT1 TIR domain is sufficient for cell death induction, we expressed individual TNT1 domains, or truncated variants in wild-type *N. benthamiana* as mYFP-tagged fusion proteins.

Expressing TNT1 without the predicted TM domain (Δ TM, TIR-NB-LRR-mYFP) failed to induce cell death (Figures 5, S8A), and CLSM shows that this variant is no longer ER localized, but shows a cytoplasmic localization (Figure 5). Microsomal fractionation, followed by immunoblot analysis, further revealed that the Δ TM variant partially lost association with the endomembrane system, in contrast to the wild-type variant containing the predicted TM domain (Figure S8B). Since the LRR domain can have an auto-inhibitory effect on HR induction (Slootweg *et al.*, 2013; Schreiber *et al.*, 2016), we expressed a construct lacking both, the TM and LRR domain (TIR-NB-mYFP). Removal of the LRR domain from the Δ TM variant shifted the localization from exclusively cytoplasmic to nucleocytoplasmic, and induced a cell death response (Figures 5, S8A and B). Cell death was also induced upon expression of the ER-associated TM-TIR-NB-mYFP and TM-TIR-mYFP, or a TIR-mYFP variant that showed strong nuclear accumulation (Figures 5, S8A and B). While the TM-TIR-NB-mYFP and TM-TIR-mYFP variants are predominantly found in the endomembrane fraction, the TIR-mYFP fusion is predominantly soluble (Figure S8B). The NB-mYFP and LRR-mYFP proteins are nucleocytoplasmic or exclusively cytoplasmic, whereas the expression of an mYFP fusion to the predicted TM domain, followed by the C-terminal flanking region (amino acids 1-43), is sufficient to anchor the protein to the ER-membrane (Figure 5, S8A and B; Lee *et al.*, 2011). No cell death was visible after expression of the NB, LRR- or TM-mYFP fusions (Figure 5).

Since the expression of truncated TNT1 versions influenced the subcellular localization and ability to trigger cell death, we also analyzed domain truncations and single domains of the TN13 protein for their localization and ability to induce cell death.

Expression of a Δ TM variant (TIR-NB-mYFP) completely abolished the localization of TN13 to the ER, but not its endomembrane association (Figure S8C-E). Whereas TM-TIR-mYFP or TM-mYFP variants showed an ER-localization, the TIR-mYFP as well as the NB-mYFP variants, did not localize to the ER, but remained partially associated with the endomembrane system (Figure S8C-E). None of the TN13 protein variants induced cell death upon transient expression in *N. benthamiana* wild-type plants (Figure S8C).

In summary, expression of the TNT1 TIR domain is sufficient to trigger a cell death response in *N. benthamiana*. The TM domain of TNT1 and TN13 is required and sufficient for the localization to the ER membrane. An ER localization of the full length TNT1 protein, but not of the TIR domain-containing truncated TNT1 protein variants, appears to be required for cell death induction (Figure 5), suggesting TM-specific suppressive effects of the LRR domain on cell death execution in *N. benthamiana*.

The predicted catalytic glutamate residue of the TNT1 TIR domain is required for cell death induction in *N. benthamiana*

A conserved catalytic glutamate (E) residue within TIR domains is required for degradation of NAD⁺/NADP⁺, leading to accumulation of cyclic ADP-ribose variants (v-cADPR) and cell death in plants (Horsefield *et al.*, 2019; Wan *et al.*, 2019; Maruta *et al.*, 2022). The TNT1 and the TN13 TIR domains both contain conserved glutamate residues at amino acid position 136 and 139, respectively (Figure, S3A). Consistent with its proposed function, mutagenesis of the TNT1 glutamate to alanine (E₁₃₆A) abolished cell death induction upon transient expression of full length TNT1, TM-TIR or TIR-only variants in *N. benthamiana* wild-type plants (Figures 6, S8F). In line with our finding that TNT1 requires the EDS1-SAG101-NRG1 module, this result suggests that TNT1 induces cell death via TIR domain enzymatic activity.

The genomic region encoding *TN13* and *TNT1* was duplicated and encodes NLRs that interact with TN13 and TNT1

The co-expression of TIR domains from TIR-type NLR pairs can suppress cell death induction of executor NLR TIR domains when expressed on their own (Williams *et al.*, 2011; Huh *et al.*, 2017). Since the overexpression of TNT1-mYFP in *Arabidopsis* does not induce autoimmunity, but triggers cell death in *N. benthamiana* (Figures 2, S2), we speculated that TNT1, TN13 and/or additional TNLs might interact with each other to suppress cell death induction in *Arabidopsis*. However, transient co-expression of TN13-mYFP and TNT1-mYFP in *N. benthamiana* did not result in a suppression of cell death (Figure S9).

Arabidopsis chromosome 3 does not encode for other NLRs within close chromosomal proximity to the *TN13/TNT1* pair (Meyers *et al.*, 2003), however, the genomic region encoding *TN13* and *TNT1* shows persistence of NLR gene presence in a segmentally duplicated chromosomal region (Simillion *et al.*, 2002; Meyers *et al.*, 2003). This chromosomal region between *AT5G14060* and *AT5G18490* on chromosome 5 was duplicated segmentally from *AT3G01015* to *AT3G04350* on chromosome 3 (Figure S10, Meyers *et al.*, 2003). The duplicated region of chromosome 5 contains an expanded number of TIR-type NLR genes, consisting of an uncharacterized genetic singleton (*AT5G17680*) and two NLR clusters (Figure 7A; based on the cluster definition by Meyers *et al.* (2003)). The first cluster encodes the head-to-head sensor-executor pair *CHS3/CONSTITUTIVE SHADE-AVOIDANCE1* (*CSA1*) (*AT5G17890/80*) which guard BAK1 and BIR3 (Schulze *et al.*, 2022) and an uncharacterized TNL (*AT5G17970*). The second cluster contains *DOMINANT SUPPRESSOR OF CAMTA3 NUMBER2* (*DSC2*, *AT5G18370*) that is required for the

camta3 autoimmune phenotype (Lolle *et al.*, 2017), *HOPB-ACTIVATED RESISTANCE1* (*BAR1*, *AT5G18360*) that recognizes the *Pseudomonas* effector HopB1 (Laflamme *et al.*, 2020), and an uncharacterized *TNL* (*AT5G18350*) in tandem orientation (Figure 7A). Phylogenetically, *AT5G18350* and *DSC2* are the closest relatives of *TNT1* and *TN13* within the duplicated chromosomal region (Figures 7B). In addition to the close phylogenetic relationship of *DSC2* to *TNT1*, *DSC2* also contains a predicted N-terminal transmembrane domain and localizes to the ER when transiently expressed in *Nbeds1a-1* plants (Figures 7B, S11, S12). The other TIR-type NLRs encoded in the duplicated region show a nuclear, cytoplasmic or nucleocytoplasmic distribution in *Nbeds1a-1* (Figure S12). Expression of all NLRs from the segmentally duplicated region on chromosome 5 triggered a cell death response in wild-type *N. benthamiana* plants, except for *CHS3* (Figure S13). Co-expression of the NLRs together with *TNT1* did not suppress cell death induction (Figure S14).

To investigate potential NLR complex formations, we conducted co-immunoprecipitation experiments after co-expression of *TN13-mYFP*, or *TNT1-mYFP*, together with 3xHA-SII tagged candidate NLRs of the duplicated region in *Nbeds1a-1* plants (including 3xHA-SII tagged versions of *TN13* and *TNT1* to monitor potential *TN13* and *TNT1* homomeric associations). Co-detection of 3xHA-SII tagged NLRs, after immunoprecipitation of mYFP-tagged proteins shows that *TNT1*, but not *TN13*, co-immunoprecipitates *BAR1* and the uncharacterized NLR encoded by *AT5G17970* (Figure 7). In addition, both *TN13* and *TNT1* can form homo-oligomers (Figure 7C) as well as hetero-oligomers with each other, and both proteins interact with *DSC2* (Figure 7C). GUS-mYFP and the CFP-tagged ER marker (ER-ck) were used as a negative control (Figure 7). As the immunoprecipitation via the C-terminal mYFP tag was inefficient for *CHS3* and *CSA1*, as well as for the uncharacterized NLRs encoded by *AT5G17680* and *AT5G18350*, no conclusion on their interaction with *TN13* and *TNT1* can be made. To independently investigate the results obtained by Co-IP for the interaction between *TN13* and *TNT1*, we employed a CLSM-based Förster resonance energy transfer-fluorescence lifetime imaging (FLIM-FRET) analysis using *TN13-mTRQ2* as donor and *TNT1-mVenus* as acceptor for fluorescence transfer. Co-expression of *TN13* with *TNT1* significantly reduced the fluorescence lifetime of *TN13-mTRQ2*, indicating *in planta* interaction (Figure 7D). A reduction in lifetime was not observed when expressing *TN13-mTRQ2* together with GUS-mVenus, used as a negative control (Figure 7D). The identified interactions and our subcellular localization studies suggest that *TN13* and *TNT1*, together with NLRs from the segmentally duplicated cluster on chromosome 5, might form a larger TIR-type NLR immune receptor network at the cytoplasmic side of the ER-membrane and the nuclear envelope in *Arabidopsis* cells.

DISCUSSION

NLRs can engage in intermolecular interactions within pairs or networks (Le Roux *et al.*, 2016; Sarris *et al.*, 2015; Césari *et al.*, 2014; Wu *et al.*, 2017; Contreras *et al.*, 2023). An additional level of complexity in TIR-type NLR function is the requirement of EDS1 family proteins and RNL-type helper NLRs for downstream signaling (Castel *et al.*, 2019; Wu *et al.*, 2019; Saile *et al.*, 2020; Wu *et al.*, 2024; Yu *et al.*, 2024). Recent examples also show the functional requirement of truncated TIR-type NLRs in immunity. Truncated TIR-NLRs were proposed to act as sensors that interact with and require an executor TIR-type NLR for efficient downstream signaling (Zhao *et al.*, 2015; Zhang *et al.*, 2017; Tong *et al.*, 2017; Liang *et al.*, 2019), albeit direct

contributions to immune signaling have also been demonstrated (Staal *et al.*, 2008; Nandety *et al.*, 2013; Nishimura *et al.*, 2017).

TN13 and TNT1 are required for full immunity to mildly virulent *P. syringae*

Our analysis revealed that *TNT1*, like *TN13*, is required for full resistance to *Pst* DC3000 (Δ AvrPto/AvrPtoB), and co-localizes with *TN13* to the ER and perinuclear membrane (Figures 1, 3, S6; Roth *et al.*, 2017). *TN13* and *TNT1* form homo-oligomers and interact with each other when co-expressed in *N. benthamiana* (Figures 7C). This suggests that *TN13* and *TNT1* may form an NLR pair involved in regulating defense responses at the ER. Since both *NLRs* are genetically required for full resistance to mildly virulent *Pst* (Figure 1), *TN13* and *TNT1* may recognize *Pst* effector(s), and initiate immune signaling to restrict growth of virulent bacteria (Thomma *et al.*, 2011). Yeast two-hybrid assays by Nandety *et al.* (2013) suggest that *TN13* interacts with the *Pst* effector HopY1 and the nematode effector Rbp001. This would be consistent with a potential sensor function of *TN13*. Notably, *BAR1* is required for recognition of the *Pst* effector HopB1 (Laflamme *et al.*, 2020). Since *BAR1* interacts with *TNT1* in *N. benthamiana* (Figure 7C), *TNT1* might also be involved in regulating HopB1-triggered defense responses.

The transient expression of *TNT1* in wild-type *N. benthamiana* triggers a cell death response that depends on *EDS1/SAG101/PAD4*, whereas no cell death is induced by *TN13* (Figures 2, S8C, S9). We hypothesize that *TNT1* could function as an executor NLR for *TN13*, which could act as a sensor lacking the capacity to produce small molecules for immune signaling on its own. Examples of TN-type sensor NLRs include *CHS1* and *TN2*, which both monitor the cellular homeostasis of the membrane-associated E3 ubiquitin ligase *SAUL1*, and signal independently via the executor *TNL* *SOC3* (Zhang *et al.*, 2017; Tong *et al.*, 2017; Liang *et al.*, 2019).

The transmembrane domains of *TN13* and *TNT1* are required for ER localization

TN13 and *TNT1* both contain N-terminal transmembrane (TM) domains that are required and sufficient to localize the mYFP-tagged fusion proteins to the ER membrane (Figures 5, S3, S6, S8A-E). Multiple alleles of the *TNL* *RPP1* from different *Arabidopsis* accessions also contain a hydrophobic extension N-terminal of the TIR domain (Schreiber *et al.*, 2016). *RPP1*-WsA localizes to ER and/or Golgi membranes, and the N-terminal extension is partially required for membrane localization and function in immunity (Weaver *et al.*, 2006). Deleting the TM-domain of *TNT1* lead to a loss of cell death when expressing this protein in *N. benthamiana* (Figure 5), which was re-established by additional deletion of the LRR domain. This probably reflects an inhibitory function of the LRR on TIR domain activity, as previously described for other NLRs (Figure 5; Sootweg *et al.*, 2013; Ntoukakis *et al.*, 2013; Schreiber *et al.*, 2016). As an ER membrane localization of TIR-containing *TNT1* truncations is not essential for cell death induction, the ER membrane association might represent an additional mechanism for regulating the activation of the full length NLR that otherwise would be suppressed by the LRR domain (Figure 5). *TNT1* cell death induction in *N. benthamiana* depends on *SAG101b/NRG1*, but not on *PAD4/ADR1* (Figure 2). Albeit such functional cooperativity is consistent with the finding that *Arabidopsis* *NRG1A* and *NRG1B* also localize to the ER (Wu *et al.*, 2019; Ibrahim *et al.*, 2024), the detailed functional interplay between *TNL* activation and *NRG1* resistosome formation at the ER remains to be explored.

Cell death induction by TNT1 requires an atypical MHV motif

The predicted catalytic glutamate residue within the TIR domain of TNT1 is required for cell death induction in *N. benthamiana* (Figure 6), suggesting that TNT1 might degrade NAD⁺/NADP⁺ to produce small molecules for the induction of immunity, as previously described for TIR domains (Wan *et al.*, 2019; Horsefield *et al.*, 2019). The TIR domains of the paired sensor TNs CHS3 and RRS1 lack the catalytic glutamate residue, enzymatic activity, and do not induce cell death in *N. benthamiana* when expressed on their own (Figure S16; Wan *et al.*, 2019; Horsefield *et al.*, 2019), whereas the TN-type sensor CHS1 contains the catalytic glutamate residue but does not induce cell death upon expression in *N. benthamiana* (Liang *et al.*, 2019). Likewise, TN13 also contains the predicted catalytic glutamate (Figures S3A, S9, S11), but expression of TN13 in *N. benthamiana* does not trigger a cell death response (Figures 2A, S8C). TN2 induced cell death in *N. benthamiana* is suppressed by co-expression of EXO70B1 (Wang, Liu, *et al.*, 2019). The RRS1-R/RPS4 sensor/executor pair, forms a higher order oligomeric complex in their inactive state, which likely requires reconfiguration upon effector interaction to derepress the RPS4 TIR domain and enable NADase enzyme function (Ahn *et al.*, 2025). It therefore remains possible that cell death induction upon expression of TN13 in *N. benthamiana* requires an additional executor as NLR signaling partner, or that cell death induction is suppressed via association with an interaction partner present in *N. benthamiana*. This might also explain why a stunted growth phenotype can be observed in the TN13 *Arabidopsis* overexpression lines, albeit no cell death is visible when expressing TN13 in *N. benthamiana* (Figure S2).

TIR domain catalytic activity depends on homo-oligomerization to form a holoenzyme resistosome (Horsefield *et al.*, 2019). Both TN13 and TNT1 show homo and hetero-oligomer formation (Figure 7). Remarkably, the TNT1 protein contains an MHV motif within the NB-ARC domain that diverges from the canonical MHD motif in other TNs and is required for cell death induction (Figures 4). TNT1 is the only *Arabidopsis* TNL containing an MHV motif within the NB-ARC domain of the wild-type protein. The presence of the MHV motif may confer higher affinity for ATP, rather than ADP, and constitutively shift TNT1 to an active state (Sukarta *et al.*, 2016). TNT1 would thus require efficient intra- or/and intermolecular repression to avoid inadvertent cell death signaling in the absence of a pathogen trigger. The CHS3/CSA1 sensor/executor pair was shown to form heterodimers and oligomerization and cell death signaling is suppressed by the receptor kinases BAK1 and BIR3, which are guarded by CHS3/CSA1 (Yang *et al.*, 2022; Schulze *et al.*, 2022; Yang *et al.*, 2024). In a similar scenario, TNT1 could function as an executor that interacts with sensors, such as TN13, and could potentially be suppressed by an unknown guard in *Arabidopsis*, but not upon transient expression in *N. benthamiana*.

The MHV motif containing TNT1 could therefore function as an integrative hub for HR induction at the interface of the ER and the cytoplasm, the PM or other organelles, that is activated upon derepression. It has previously been reported that expression of *TNT1* is upregulated following elicitor treatment, *Pseudomonas* infection, or in autoactive mutant plants exhibiting constitutive defence responses (Tian *et al.*, 2021; Lang *et al.*, 2022). In addition, upregulation of *TNT1* expression is dependent on MPK3/6 kinase signaling and induced expression of *TNT1* activates salicylic acid biosynthesis genes (Lang *et al.*, 2022). Therefore, TNT1 might also contribute to the induction of basal defense which would explain the defects of the *tnt1* mutant in basal resistance to *Pst* DC3000 (Δ AvrPto/AvrPtoB).

TN13 and TNT1 interact with each other and NLRs in a segmentally duplicated genomic region

The region of chromosome 3 containing *TN13-TNT1* was duplicated segmentally to chromosome 5 (Figures 7, S10; Simillion *et al.*, 2002; Meyers *et al.*, 2003). The duplicated region on chromosome 5 contains syntenic regions with an expanded number of *NLR* genes (Meyers *et al.*, 2003, Figures 7, S10). Phylogenetic analysis of the complete repertoire of *Arabidopsis* (Col-0) TIR-type NLRs suggest that TN13 and TNT1 are closely related to NLRs of the tandem cluster, including AT5G17970 (Figure 7; Van de Weyer *et al.*, 2019). This is expected based on a model of conserved synteny, where physical proximity correlates with close phylogenetic relationship (Baumgarten *et al.*, 2003) and would further support that the tandem cluster components *AT5G18350/BAR1/DSC2*, and potentially *AT5G17970*, originated from *TN13* and/or *TNT1*. It has been proposed that tandem gene duplications play a major role in the evolution of plant NLRs, while segmental duplications are less prevalent, but partially explain the expansion of *NLRs* within the *Arabidopsis* genome to distinct chromosomal regions (Richly *et al.*, 2002; Leister, 2004; Cannon *et al.*, 2004).

The duplication of *NLRs* can be considered as a mechanism to generate diversification, to produce new NLR variants for effector detection (Leister, 2004; Cannon *et al.*, 2004; Barragan and Weigel, 2021). All *NLRs* within the segmentally duplicated clusters of chromosome 3 and 5 belong to the *Arabidopsis* core NLRome, with the exceptions of *TN13*, *AT5G17970* and *AT5G18350* that are part of the shell NLRome and potentially arose via tandem duplication (Figure S14; Van de Weyer *et al.*, 2019). Expanded tandem duplicated regions, such as the *RPP1* cluster, were also identified as *DANGEROUS MIX (DM)* loci. These loci can lead to hybrid necrosis upon crossing of different accessions due to NLR repertoire incompatibility caused by diversifying pressure on loci in the *NLR* clusters (Bomblies *et al.*, 2007; Chae *et al.*, 2014; Alcázar *et al.*, 2014; Stuttmann *et al.*, 2016; Atanasov *et al.*, 2018).

Both TN13 and TNT1 interact with DSC2 (AT5G18370). In addition, TNT1 interacts with BAR1 (AT5G18360) and the uncharacterized TNL protein encoded by *AT5G17970* (Figure 7). These findings suggests that TN13 and TNT1 could be part of a larger TNL signaling network that involves TNLs from the duplicated cluster. The hetero-pairing of TN13 and TNT1 with NLRs of the duplicated cluster on chromosome 5 might be explained by their close phylogenetic relation. In fact, several sensor-executor NLR pairs that are genomically linked fall into neighboring phylogenetic sub-clades (Césari *et al.*, 2014; Van de Weyer *et al.*, 2019; Ahn *et al.*, 2025). Since *tn13* mutation as well as *TNT1* over-expression in stable transgenic plants did not show an auto-immune phenotype (Figure S2), we speculate that TNT1 is repressed by additional TNLs or other host proteins. Most NLRs encoded in the duplicated cluster contain a predicted catalytic glutamate residue within the TIR domain and trigger a cell death response when expressed individually, or together with TNT1, in *N. benthamiana* (Figures S12, S13). Whether TNLs of the duplicated region function together with TNT1 or potentially influence cell death induction of TNT1 upon co-expression of multiple TNL combinations, remains to be investigated.

EXPERIMENTAL PROCEDURES

Plant materials

All plants were grown in environmentally controlled growth chambers with 65 % relative humidity. *Arabidopsis thaliana* plants were grown on soil for infection studies, or on ½ MS agar-plates for microscopy analyses, under an 8/16 h light/dark regime at

22/18°C. *Nicotiana benthamiana* plants were grown under a 16/8 h light/dark regime at 25/22°C. The *N. benthamiana eds1a-1* mutants (Ordon *et al.*, 2017), *pad4* and *sag101a/b* (single, double and triple) mutant combinations (Gantner *et al.*, 2019; Lapin *et al.*, 2019), as well as the *Arabidopsis tn13* (Roth *et al.*, 2017), *snc1* (Li *et al.*, 2001) and Col *eds1-2* (Bartsch *et al.*, 2006) mutants were previously described. The *N. benthamiana adr1 nrg1* and *nrg1* mutant lines were kindly provided by Jane Parker (MPIPZ, Cologne) and Johannes Stuttmann (BIAM - CEA Cadarache). The *tnt1-1* mutant line were obtained from the Nottingham Arabidopsis Stock Centre (NASC, <http://arabidopsis.info>). Homozygous mutants were isolated via PCR-based genotyping, using primers listed in Table S1. Stable transgenic *Arabidopsis* plants were generated by using the floral dip method (Clough and Bent, 1998).

Plasmid constructions

The full-length genomic sequences (with or without promoter region) were PCR amplified without stop codon, using gene specific primers listed in Table S1, and cloned into pENTR/D-TOPO (ThermoFisher Scientific, <https://www.thermofisher.com>) Gateway entry vectors. The generation of truncations, or site-directed mutagenesis, was conducted by sub-cloning into pENTR/D-TOPO and/or mutagenesis PCR (Q5® Site-Directed Mutagenesis Kit, NEB), using primers listed in Table S1 and respective pENTR/D-TOPO entry clones as templates. The pENTR/D-TOPO *GUS+intron* and *TN13* entry vectors (Roth *et al.*, 2017), the *ER-ck* (Nelson *et al.*, 2007), and *NRG1* (Lapin *et al.*, 2019) expression vectors were described previously. All expression constructs were generated via Gateway LR-reactions with pXCSG-GW-mYFP, pXCG-GW-mYFP or pXCSG-GW-3xHA-StrepII destination vectors (Witte *et al.*, 2004; Feys *et al.*, 2005) and respective pENTR/D-TOPO sequence-verified entry vectors. All generated expression vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 pMP90RK (Koncz and Schell, 1986), using electroporation.

Pseudomonas infection assays

A bacterial suspension of *Pst* DC3000 (Δ AvrPto/AvrPtoB; Lin and Martin, 2005) with a density of 1×10^5 cfu ml⁻¹ in 10 mM MgCl₂ was infiltrated into rosette leaves of five 5-weeks-old soil grown *Arabidopsis* plants, using a needleless syringe. Colony forming units were determined 1 h (d0) and 3 days (d3) after infiltration.

Transient expression in *N. benthamiana*

Agrobacterium overnight cultures were harvested by centrifugation, resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES pH 5.5, 150 μ M acetosyringone), and incubated for 2-3 h at room temperature. For co-expressions, the bacterial suspension was adjusted to an OD₆₀₀ of 0.3 for each strain and infiltrated into the abaxial side of 4-5 weeks old *N. benthamiana* leaves, using a needleless syringe. An *Agrobacterium* strain expressing the silencing suppressor p19 was co-infiltrated in all transient expression experiments. For HR cell death assays, the infiltrated leaf areas were photographed 5 days after infiltration. Confocal laser scanning microscopy was performed 2 days after *Agrobacterium* infiltration.

Confocal laser scanning microscopy

For confocal microscopy, leaf discs of *Agrobacterium*-infiltrated *N. benthamiana* tissues or stable transgenic *Arabidopsis* plants were embedded in water. A Leica TSC-SP5 confocal laser-scanning microscope controlled by the Leica LAS AF software was used with a 20x/0.70 objective (PL APO, CS). An argon laser line at 514 nm was used

for excitation of mYFP, and 458 nm for CFP. Emitted fluorescence was detected at 525 – 570 nm for mYFP, and 465 – 485 nm for CFP, using Leica HyD detectors. Chlorophyll auto-fluorescence was detected at 680–720 nm, using a Leica PMT detector. All channels were scanned sequentially. ImageJ was used to merge channels (Schindelin *et al.*, 2012). FLIM-FRET experiments were conducted with transiently transformed *N. benthamiana* leaves using a Leica TCS SP8 microscope equipped with the FALCON time-correlated single photon counting system. The mTurquoise2 (mTRQ2)-tagged donor proteins were excited using a pulsed diode laser at 440 nm, and the mVenus-tagged acceptor proteins were excited with a pulsed white light laser at 514 nm. A pulse rate of 40 kHz and a two-channel sequential excitation were applied. The fluorescence emission was detected at 465-505 nm for mTRQ2 and 520-560 nm for mVenus using HyD SMD detectors. Images were acquired when at least 100 photons/pixel were collected in the brightest channel. The picture format was set to 512 x 512 pixels. Selection of regions of interest and FLIM data fitting were performed using the LAS X Single Molecule Detection software module (v3.5.5). For fluorescence lifetime calculations, a mono-exponential re-convolution fitting model was applied. Statistical analysis and plotting of the donor lifetime data was performed in Origin 2020 (OriginLab Corp., Northampton, USA).

Total protein extracts, co-immunoprecipitation, microsomal fractionation and immunoblot analysis

To generate total protein extracts from *Arabidopsis* samples, leaf tissues were frozen in liquid nitrogen, homogenized and mixed with protein extraction buffer (250 mM sucrose, 100 mM HEPES-KOH (pH 7.5), 5 % (v/v) glycerol, 2 mM Na₂MoO₄, 25 mM NaF, 10 mM EDTA, 1 mM DTT, 0.5 % (v/v) Triton X-100, plant protease inhibitor cocktail (#P9599, Sigma)), cell debris was removed by centrifugation and filtration through a 95 µm nylon mesh. Protein concentrations were quantified using Bradford reagent (Biorad). Protein samples were adjusted to equal concentrations, mixed with 4x SDS-PAGE loading dye (250 mM Tris-HCl (pH 6.8), 8 % (w/v) SDS, 40 % (v/v) Glycerol, 0.04 % (w/v) Bromophenol blue, 400 mM DTT) and boiled. For co-immunoprecipitation analysis (Co-IP) after *Agrobacterium*-mediated transient expression in *N. benthamiana*, 7.5 µl GFP-trap magnetic agarose beads (Chromotek) were equilibrated in protein extraction buffer, and added to each total protein extract generated as described for *Arabidopsis* samples, without adjusting total protein concentrations. Incubation was performed for 3 h at 4 °C under constant rotation before the magnetic agarose beads were isolated and washed 3 times in 1 ml extraction buffer, using a magnetic rack. Proteins were eluted by boiling in 4x SDS loading dye. For microsomal fractionation after *Agrobacterium*-mediated transient expression in *N. benthamiana*, total protein extracts were prepared as described for *Arabidopsis* samples, without adjusting total protein concentrations, using a buffer without Triton X-100. Total protein extracts were subjected to ultra-centrifugation at 100.000 g, and an aliquot of the supernatant (soluble fraction) was mixed with 4x SDS loading dye. The endomembrane pellet was washed 3 times in 1 ml extraction buffer and dissolved in extraction buffer containing 0.5 % (v/v) Triton X-100, before mixing with 4x SDS loading dye and boiling. For total protein extraction from *N. benthamiana*, leaf tissues were frozen in liquid nitrogen, homogenized and boiled in 2x SDS-PAGE loading dye. Cell debris was removed by centrifugation at 17.000 g, and the supernatant was used for SDS-PAGE and immunoblot analysis. All protein samples were separated on SDS polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham Protran, 0.45 µm; GE Healthcare Life Sciences). Primary α-

GFP (#11814460001, Roche, 1:5000), α -HA (H9658; Sigma-Aldrich, 1:5000), and α -PEPC (100-4163, Rockland, 1:7500) antibodies and secondary goat anti-mouse IgG-poly-HRP (polyclonal, #32230; ThermoFisher Scientific, 1:5000) or goat anti-rabbit IgG-poly-HRP (polyclonal, #32260; ThermoFisher Scientific, 1:5000) antibodies were incubated and detected using SuperSignal West Femto chemiluminescence substrate (#34095; ThermoFisher Scientific) on a ChemiDoc imaging system (BioRad).

RNA isolation and RT-PCR analysis

Isolation of total RNA and RT-PCR analyses were conducted as described by Genencher *et al.*, (2016), using primers listed in Table S1 in a 20 μ l reaction volume. HDGreen (Intas) was used for staining of the gel.

DNA isolation and genotyping PCR

Isolation of total genomic DNA was performed using the CTAB method. Briefly, 100 mg of *Arabidopsis* leaf tissue was ground to a fine powder in liquid nitrogen using a TissueLyser (QIAGEN, Hilden, Germany). CTAB extraction buffer was added, mixed and incubated for 20min at 65 °C. Chloroform was added and the aqueous phase was mixed with double volume Ethanol. The DNA was precipitated at -20 °C for 30 min and pelleted by centrifugation. DNA was dissolved in ddH₂O and standard PCR genotyping was performed using primers listed in Table S1. HDGreen (Intas) was used for staining of the agarose gel.

Phylogenetic analyses

For phylogenetic analyses, the Araport11 protein sequences of the Col-0 NLRome (based on Lee and Chae, 2020) were retrieved by providing a list of the AGI locus IDs in the TAIR gene search function (<https://www.arabidopsis.org/search/genes>). Domain predictions were performed in Geneious Prime (Kearse *et al.*, 2012) using InterProScan5 (Jones *et al.*, 2014), Transmembrane domains were predicted using TMHMM (Krogh *et al.*, 2001). The NB-ARC and Winged-Helix domains of NLRs were extracted from an alignment, based on annotations. The extracted domains were re-aligned and a phylogenetic tree was generated. Alignments were performed using MAFFT (Kato *et al.*, 2002; Kato and Standley, 2013), FastTree (Price *et al.*, 2010) was used to generate phylogenetic trees. An alignment of the NB-ARC domains and the phylogenetic tree file are available as Supplementary Dataset S3 under <https://zenodo.org/records/15281087>.

Motif frequency analysis

For MHD motif frequency analysis an alignment of deduplicated NLRs from 180 RefSeq reference plant proteomes, containing only the NB-ARC domains (Toghiani and Kamoun, 2024; Ibrahim *et al.*, 2024), was manually trimmed to the MHD motif using Geneious Prime (Kearse *et al.*, 2012). The trimmed file was used to determine the frequency for each amino acid in each position. The trimmed MHD motifs are available as Supplementary Dataset S2 under <https://zenodo.org/records/15281087>.

Statistical analysis

All statistical analyses were performed using R, v 2024.12.1+563 or Origin 2020 (OriginLab Corp., Northampton, USA). A Kruskal-Wallis test, followed by Pairwise Wilcoxon tests with Benjamini-Hochberg correction were performed on non-parametric data for pathogen infection assays after log transformation. For FRET-

FLIM analysis, one-way ANOVA coupled with Tukey's test ($p < 0.001$) was used on donor lifetime.

Structural prediction

AlphaFold3 (Abramson *et al.*, 2024) was used with a seed value of 1 to generate structural models of the TN13 and TNT1 TM-TIR domains. The predicted structures were analyzed in ChimeraX (Pettersen *et al.*, 2021), where domains were colored according to their pLDDT scores. The PDB files of the structural models are available as Supplementary Dataset S1 under <https://zenodo.org/records/15281087>.

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CONFLICT OF INTEREST

None of the authors has declared a conflict of interest.

SUPPORTING INFORMATION

Figure S1. Genotyping of *tnt1-1* and *tn13 tnt1* mutant lines.

Figure S2. Phenotyping of *tn13* and *tnt1* mutant and transgenic complementation lines.

Figure S3. Predicted domain structures and functional NLR motifs of TNT1 and TN13.

Figure S4. TNT1-mYFP protein accumulation upon *Agrobacterium*-mediated transient expression in *N. benthamiana eds1a-1* mutant lines.

Figure S5. Localization of TN13-mYFP and TNT1-mYFP upon *Agrobacterium*-mediated transient expression in *Nbeds1a-1* leaves under control of the respective native promoters.

Figure S6. Localization of TN13-mYFP and TNT1-mYFP in stable transgenic *Arabidopsis* lines, expressing the fusion protein constructs under control of the 35S promoter.

Figure S7. Immunoblot analysis of total protein extracts, derived from tissues transiently expressing TNT1-mYFP wild-type and mutant variants.

Figure S8. NLR domain truncation and mutation analysis.

Figure S9. *Agrobacterium*-mediated transient co-expression of TN13-mYFP and TNT1-mYFP in wild-type *N. benthamiana* does not suppress TNT1 induced cell death.

Figure S10. Dot plot matrix analysis of genomic regions.

Figure S11. Protein sequence alignment of NLRs within the segmentally duplicated regions of chromosome 3 and 5.

Figure S12. Localization of mYFP-tagged NLRs encoded in the segmentally duplicated cluster on chromosome 5.

Figure S13. Cell death induction upon transient expression of NLRs encoded in the segmentally duplicated cluster on chromosome 5.

Figure S14. NLR presence analysis within *Arabidopsis* accessions.

Table S1. Primers used in this study.

Dataset S1. AlphaFold3 generated PDB files of TN13 and TNT1 TM-TIR domains.

Dataset S2. Amino acids of the MHD motif extracted from a RefSeq NLR database.

Dataset S3. Alignment and phylogenetic tree files of *Arabidopsis* Col-0 NLRs.

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FIGURES

Figure 1

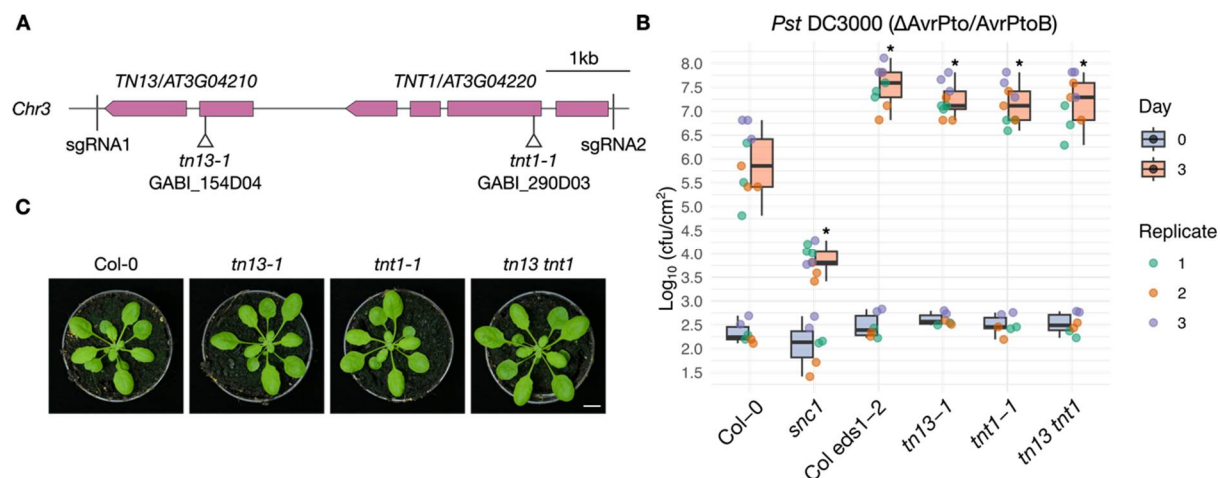


Figure 1. *TNT1* and *TN13* mutants show enhanced susceptibility to *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 ($\Delta\text{AvrPto}/\text{AvrPtoB}$). (A) Gene structures of genetically linked *TN13* and *TNT1* drawn to scale. Exons are represented as boxes and introns as solid lines. The positions of the T-DNA insertions in mutant lines used in infection studies are marked by triangles below the gene structures. The position of single guide RNAs (sgRNA) used to generate the *tn13 tn1* double mutant are indicated. (B) Plants of the indicated genotypes were infiltrated with a *Pst* DC3000 ($\Delta\text{AvrPto}/\text{AvrPtoB}$) suspension of 1×10^5 cfu ml^{-1} . Colony-forming units (cfu) were quantified 1 hour (day 0) and three days after infiltration (day 3). Data is presented as boxplots, datapoints are colored based on replicates. Asterisks indicate statistically significant differences to Col-0 (Kruskal-Wallis test; Pairwise Wilcoxon tests with Benjamini-Hochberg correction, $P < 0.05$). (C) Representative images of four-week-old plants of the indicated genotypes grown in parallel on soil under short day (SD) conditions. Scale bar = 1 cm.

Figure 2

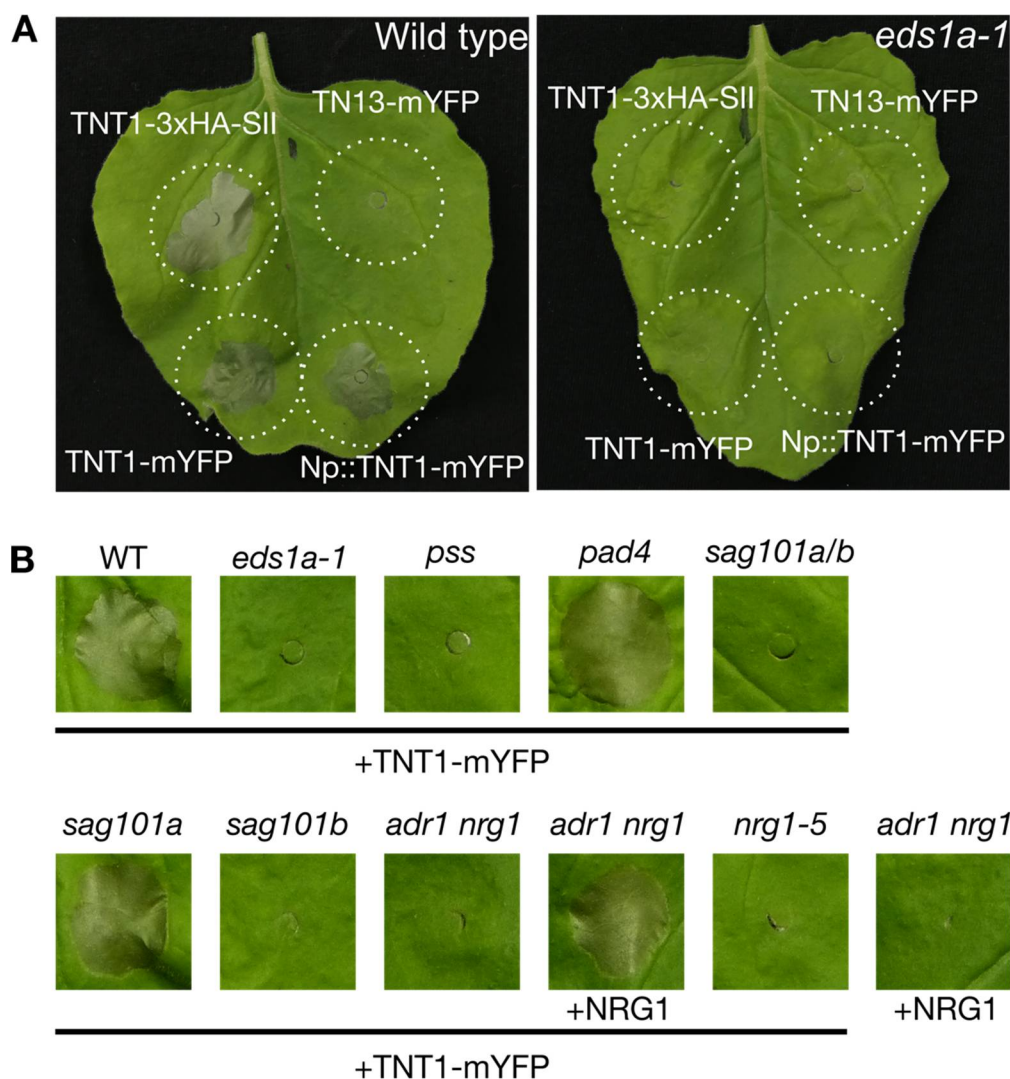


Figure 2. Transient expression of TNT1 in *N. benthamiana* induces a cell death response that depends on *NbEDS1*, *NbSAG101b* and *NbNRG1*. (A) *N. benthamiana* plants of the indicated genotypes were photographed 5d after infiltration with *Agrobacteria* for transient expression of mYFP or 3xHA-StrepII (3xHA-SII) tagged versions of TNT1 or TN13, under control of the 35S or native (*Np*) promoter. (B) *N. benthamiana* plants of the indicated genotypes were infiltrated with *Agrobacteria* for expression of TNT1-mYFP under control of the 35S promoter. The *adr1 nrg1* double mutant was complemented with *NRG1* via transient co-expression. *pss*: *pad4 sag101a sag101b*. Cell death was photographed 5d after *Agrobacterium* infiltration.

Figure 3

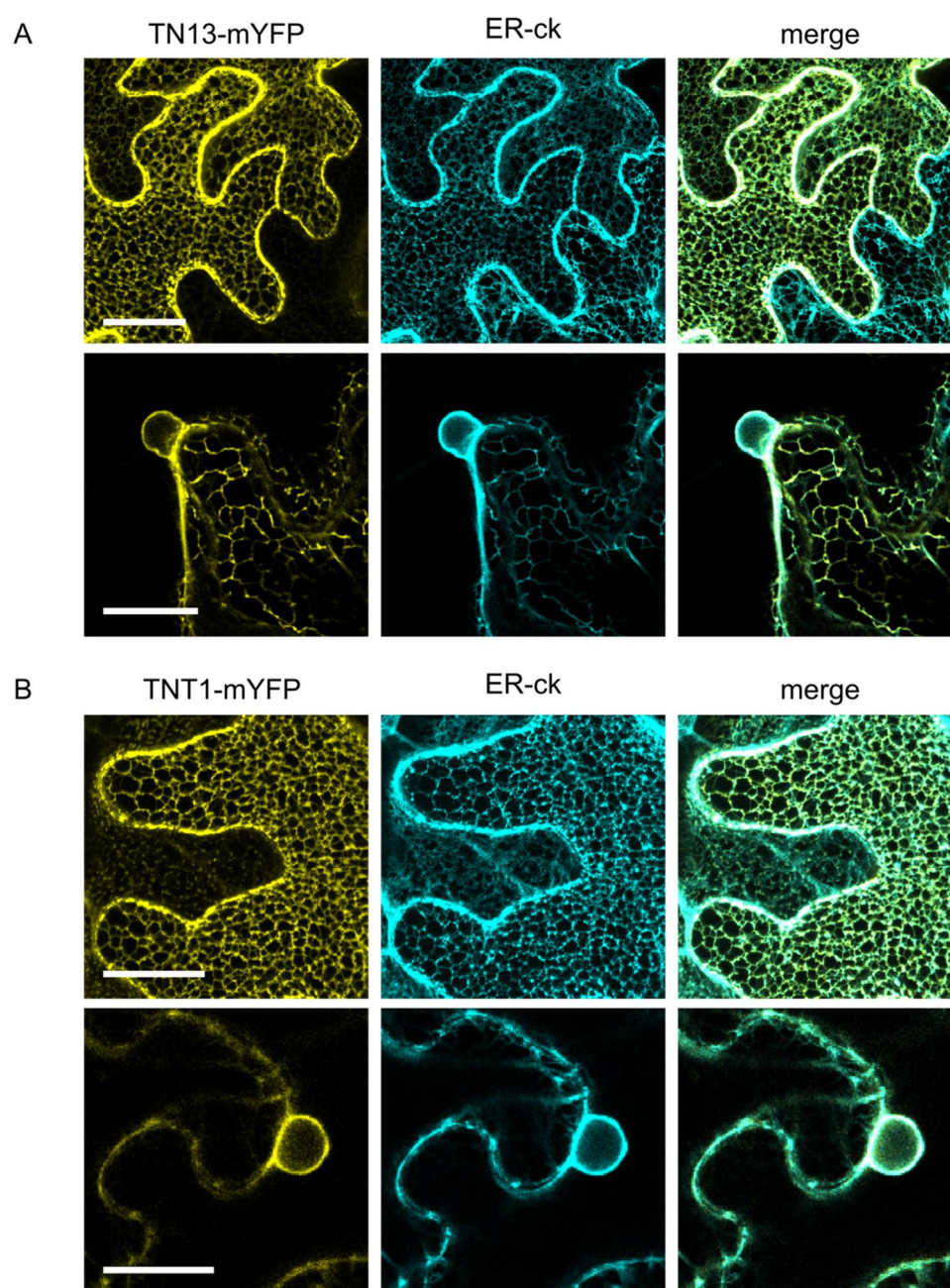


Figure 3. TN13-mYFP and TNT1-mYFP are ER membrane localized proteins. Localization of TN13 (A) and TNT1 (B) upon *Agrobacterium*-mediated transient expression in *Nbeds1a-1* leaves under control of the 35S promoter. Confocal laser scanning microscopy was performed 2 days after *Agrobacterium* infiltrations. The top panels in (A) and (B) show z-stacks, the panels below show a single confocal plane crossing the nucleus. TN13-mYFP and TNT1-mYFP are shown in yellow, the co-expressed CFP-tagged ER marker (ER-ck, Nelson *et al.*, 2005) is shown in cyan. Scale bar = 25 μm.

Figure 4

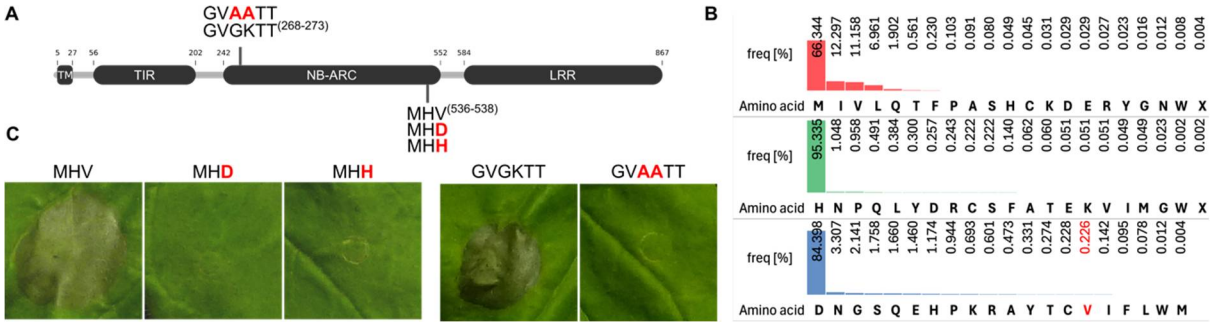


Figure 4. Cell death induction by TNT1 depends on the atypical MHV-motif and a functional P-loop. (A) Schematic representation of the TNT1 protein domain structure indicated by the amino acid positions within the TNT1 sequence. Amino acid exchanges introduced by site-directed mutagenesis are indicated in red. (B) Frequency of amino acids in each position of the MHD motif based on data of 56,686 NLRs from the RefSeq NLR database (Toghiani and Kamoun, 2024; Ibrahim *et al.*, 2024). X indicates ambiguous amino acid. (C) *Agrobacterium*-mediated transient expression of the indicated TNT1 wild-type and mutant variants fused to mYFP in *N. benthamiana* wild-type plants under control of the 35S promoter. Cell death was photographed 5d after *Agrobacterium* infiltration.

Figure 5

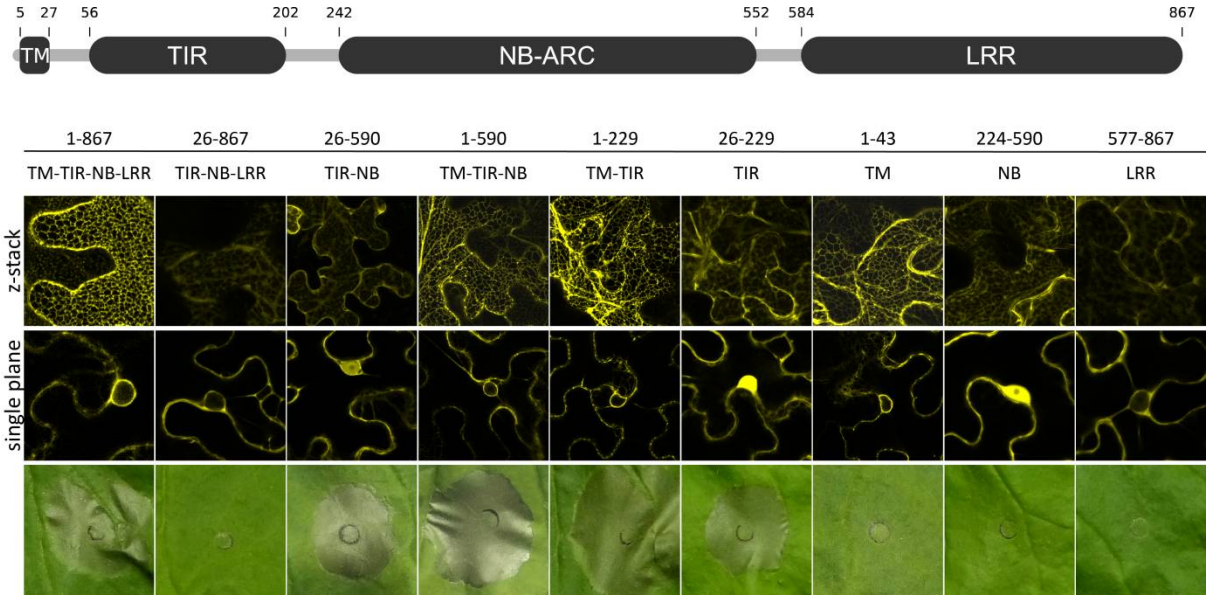


Figure 5. The TIR domain of TNT1 is sufficient for cell death induction. A schematic overview of the predicted TNT1 protein domain structure, with beginning and end of the respective domains indicated by amino acid positions at the top. The amino acids and domains of TNT1 expressed as mYFP fusions are indicated above CLSM images showing z-stacks (upper panel), and single confocal planes (middle panel) crossing the nucleus. Confocal images were taken 2 d after *Agrobacterium*-infiltration of *Nbeds1a-1* leaves. Transient expression for cell death assays (bottom panel) was performed in leaves of *N. benthamiana* wild-type plants, infiltrated leaf areas were photographed 5 d after *Agrobacterium* infiltration. Confocal images showing the localization of the full length TNT1 protein are also displayed in Figure 3B.

Figure 6

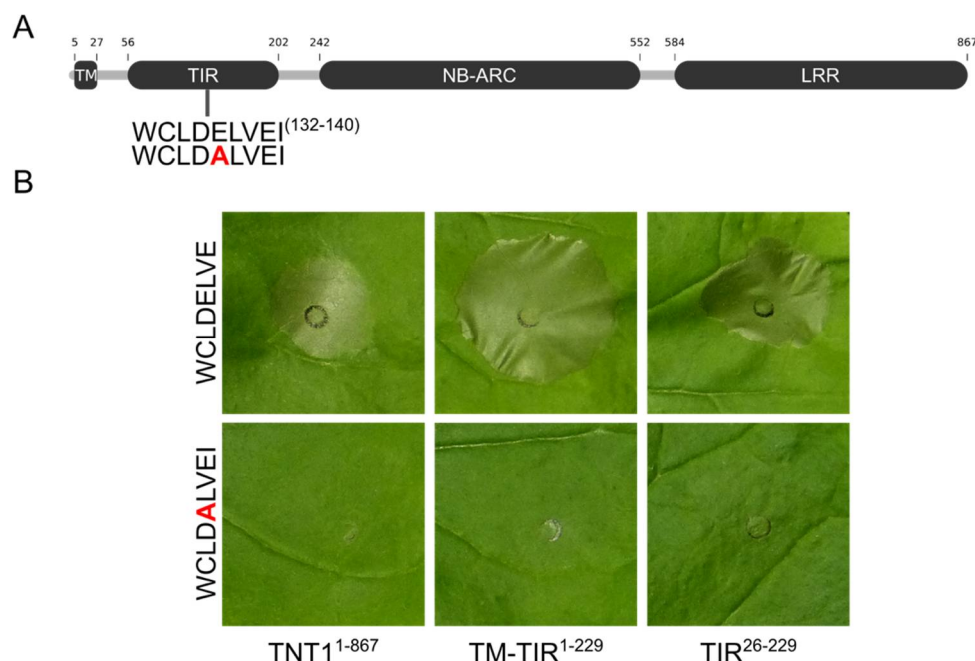


Figure 6. Cell death induction of TNT1 in *N. benthamiana* wild-type plants depends on the predicted catalytic glutamate residue of the TIR domain. (A) Schematic representation of the TNT1 protein domain structure. The predicted beginning and end of the respective protein domains are indicated by amino acid positions at the top. Amino acids 132-140 of the predicted catalytic motif in the TIR domain, containing the essential catalytic glutamate residue (E₁₃₆), are depicted in single letter code. The amino acid exchange introduced via site-directed mutagenesis is indicated in red (A and B). (B) *Agrobacterium*-mediated transient expression of the indicated TNT1 wild-type and E₁₃₆A mutant variants in *N. benthamiana* wild-type plants under control of the 35S promoter. Cell death was photographed 5d after *Agrobacterium* infiltration.

Figure 7

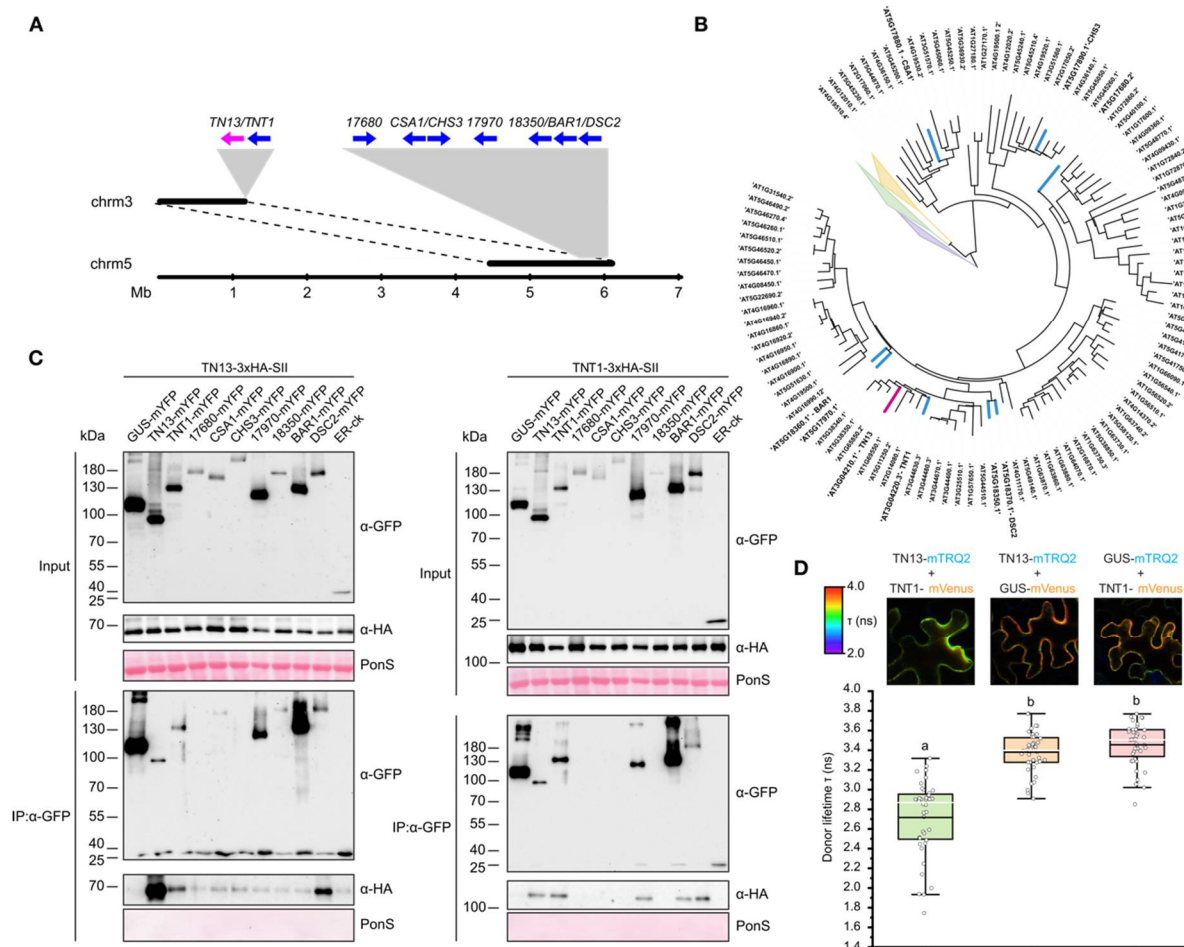


Figure 7. TN13 and TNT1 interact with each other and with NLRs encoded by a segmentally duplicated genomic region on *Arabidopsis* chromosome 5. (A) Schematic representation of the position of the segmental duplication of chromosome (chr) 3 and chromosome 5. Full length *TIR*-type *NLR*s are depicted as blue arrows, *TN*-type *NLR*s in magenta. The corresponding gene names or loci are indicated. (B) Phylogenetic tree of *Arabidopsis* *NLR*s based on the NB-ARC domain. RNL (purple), CCG10 (yellow), and CNL (green) clades are collapsed. (C) mYFP-tagged proteins were immunoprecipitated from protein extracts obtained 2 days after *Agrobacterium*-infiltration for transient expression of the indicated fusion proteins in *Nbeds1a-1* plants, using GFP-Trap magnetic agarose beads (IP: α-GFP). Co-immunoprecipitation of 3xHA-StrepII (3xHA-SII)-tagged *NLR*s was detected by α-HA immunoblots. Total protein extracts (Input) were probed with α-GFP and α-HA antibodies, respectively. GUS-mYFP and a CFP-tagged ER marker (ER-ck; Nelson *et al.*, 2007) served as negative controls. All samples were separated on 7.5 % SDS polyacrylamide gels, blotted onto nitrocellulose membranes and probed with the indicated primary antibodies. Horseradish peroxidase coupled secondary antibodies were used for detection. PonceauS (PonS) staining of the membranes was used as loading control. (D) FLIM measurements of transiently expressed donor proteins TN13-mTRQ2 or GUS-mTRQ2 in *N. benthamiana eds1a-1* epidermal cells, two days post-infiltration (dpi) of *Agrobacteria* for co-expression of the fusion proteins indicated on top. Donor lifetime heat maps of representative cell areas used for FLIM measurements are shown according to the scale on the left. Data are presented as box plots. Dots represent individual measurements, the lower and upper limits of the box indicate the interquartile range, and the black and white lines represent the mean and median,

respectively. Different letters above the plots indicate a statistically significant difference among different groups (n=40, one-way ANOVA, Tukey's test, $P < 0.001$).

Figure S1

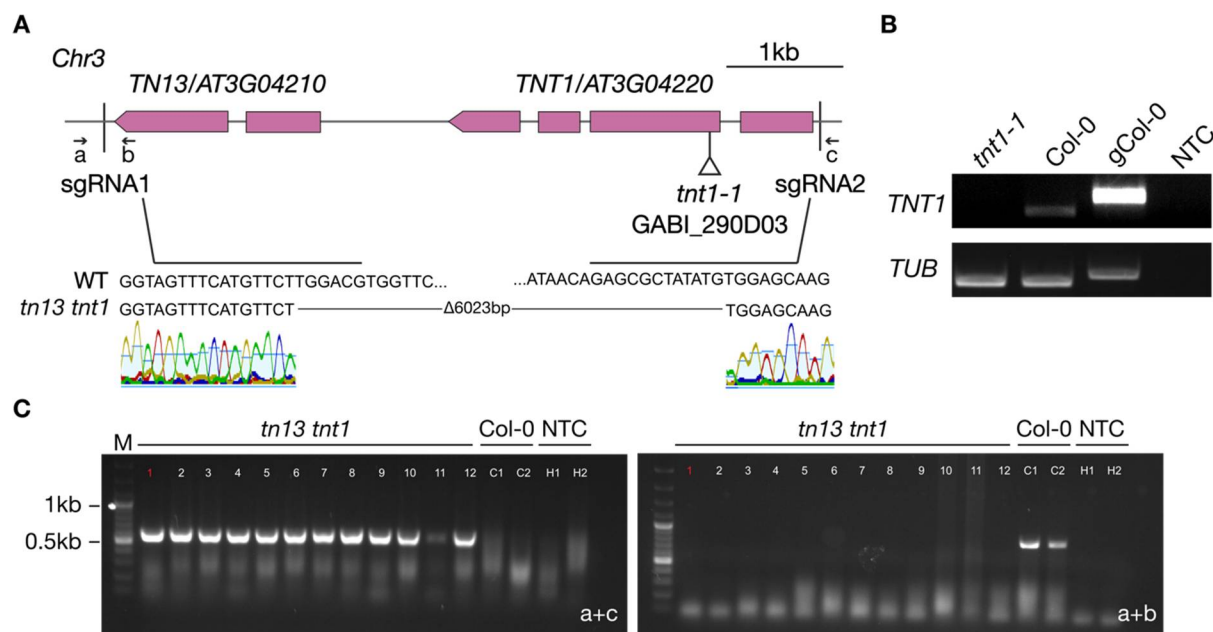
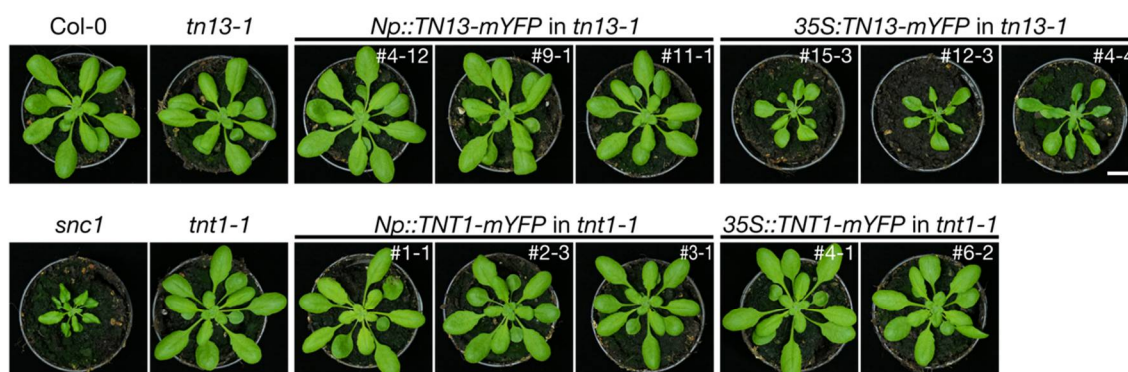


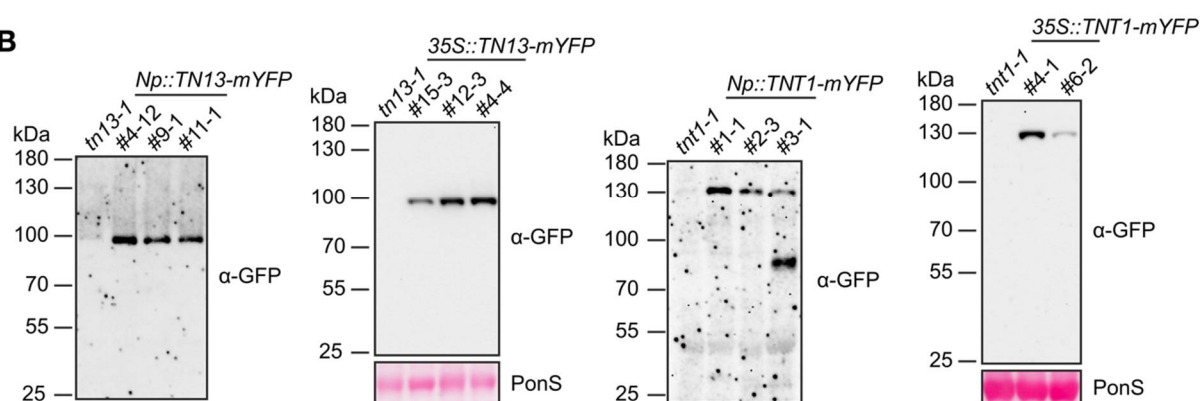
Figure S1. Genotyping of *tnt1-1* and *tn13 tnt1* mutant lines. (A) Gene structures of genetically linked *TN13* and *TNT1* drawn to scale. Exons are represented as boxes and introns as solid lines. The positions of the T-DNA insertion in *tnt1-1* is marked by a triangle below the gene structure. The position of single guide RNAs (sgRNA) used to generate the *tn13 tnt1* double mutant are indicated. Flanking (a+c) and gene specific (a+b) primers used for genotyping of the *tn13 tnt1* double mutant are indicated. (B) RT-PCR analysis with cDNAs, transcribed from RNA of four weeks old *tnt1-1* T-DNA insertion lines, grown on soil under short day conditions. *TUBULIN* (*TUB*) was used as control. PCR products were separated by agarose gel electrophoresis and stained by HDGreen. (C) PCR-genotyping of the *tn13 tnt1* double mutant. Standard PCR was performed with primers flanking the single guide RNA (sgRNA) targets sites and gene specific primers to detect gene deletions. PCR products were separated by agarose gel electrophoresis and stained by HDGreen. C1/2 and H1/2 represent Col-0 and ddH₂O controls, respectively. The red number above the band indicates the line used in subsequent experiments.

Figure S2

A



B



C

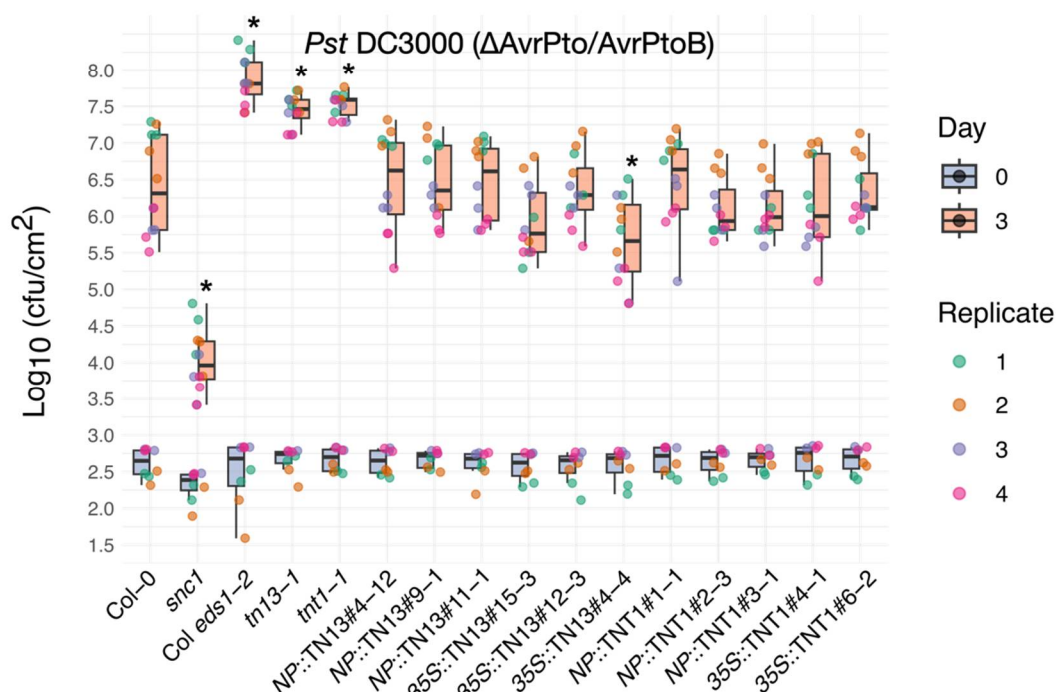


Figure S2. Phenotyping of *tn13* and *tn1* mutant and transgenic complementation lines. (A) Representative images of four-weeks-old plants of the indicated genotypes grown in parallel on soil under short day (SD) conditions. Scale bar = 1 cm. (B) Immunoblot analysis of GFP-trap magnetic agarose beads (Chromotek) immunoprecipitated protein from *Np* driven complementation lines and total protein extracts from *35S* driven complementation lines, derived from plants of the indicated genotypes. Samples were separated on a 7.5 % SDS polyacryamide gel, blotted onto a nitrocellulose membrane and probed with α -GFP primary antibody and horseradish peroxidase coupled secondary antibody. Loading for total protein extracts was monitored by PonceauS (PonsS) staining of the membrane. (C) Plants of the indicated genotypes were infiltrated with a *Pst* DC3000 (Δ AvrPto/AvrPtoB) suspension of 1×10^5 cfu ml⁻¹. Colony-forming units (cfu) were quantified 1 hour (day 0) and three days after infiltration (day 3). Data is presented as boxplots, datapoints are colored based on replicates. Asterisks indicate statistically significant differences to Col-0 (Kruskal-Wallis test; Pairwise Wilcoxon tests with Benjamini-Hochberg correction, $P < 0.05$).

Figure S3

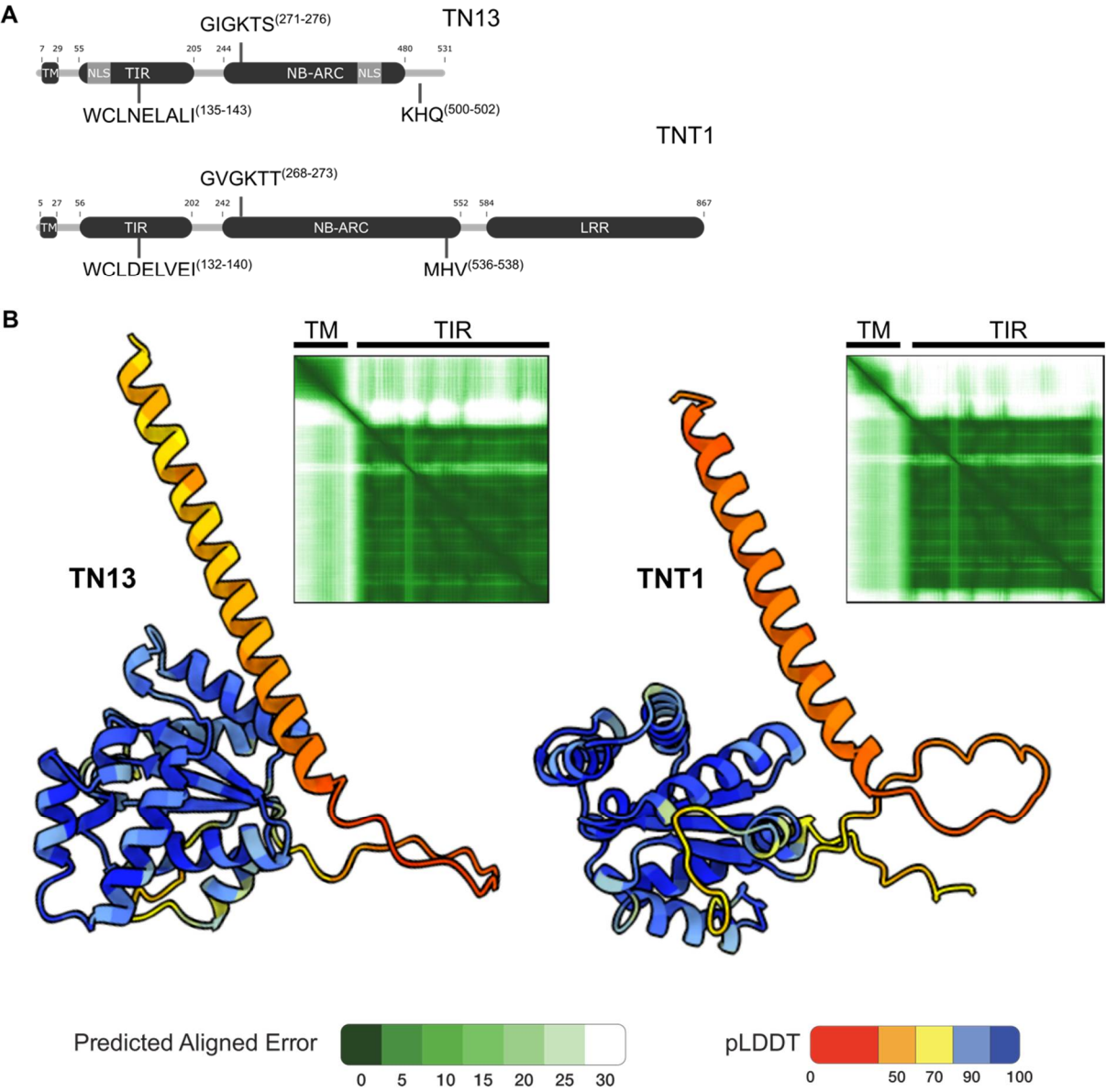


Figure S3. (A) Predicted domain structures and functional NLR motifs detected in TN13 and TNT1. The relative position of the TM, TIR and NB-ARC domains of TN13 and TNT1 are indicated. (B) AlphaFold3 prediction of the N-terminal transmembrane (TM) and TIR domains of TN13 and TNT1. The predicted Local Distance Difference Test (pLDDT) as measure of per-residue local confidence, and the Predicted Aligned Error (PAE) to display the relative expected positional error for each residue are shown.

Figure S4

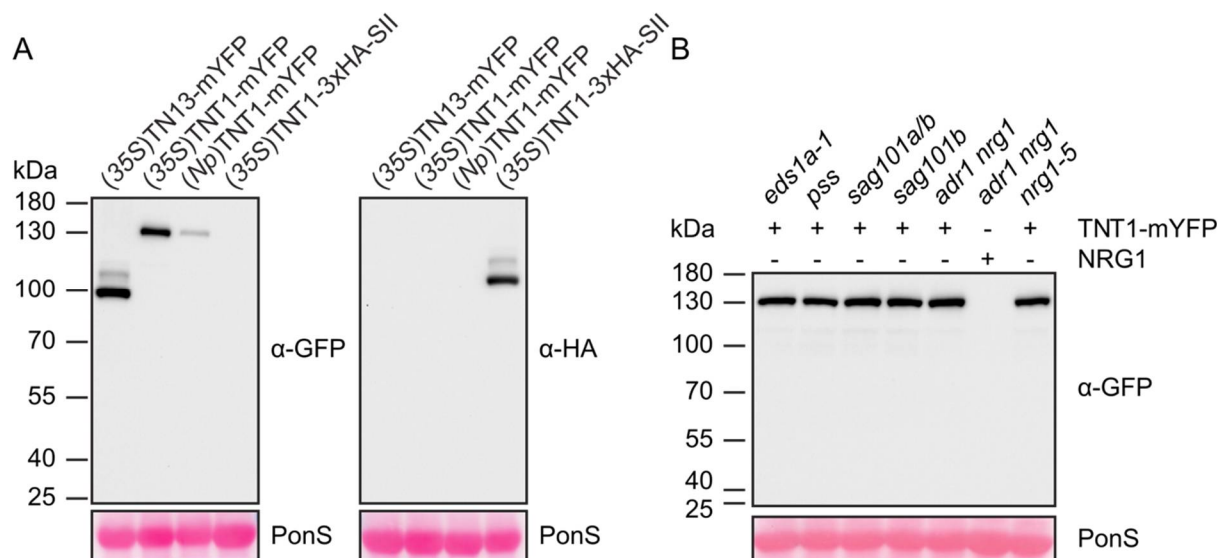


Figure S4. TNT1-mYFP protein accumulation upon *Agrobacterium*-mediated transient expression in *N. benthamiana* *eds1-1* mutant lines. (A) Immunoblot analysis of total protein extracts of *N. benthamiana* *eds1-1* leaf tissues, expressing TN13-mYFP, TNT1-mYFP or TNT1-3xHA-SII under control of the native (*Np*) or 35S promoter. (B) Immunoblot analysis of total protein extracts from tissues of the indicated mutant lines, 5 days after infiltration of *Agrobacterium*. Samples were separated on a 7.5 % SDS polyacrylamide gel, blotted onto nitrocellulose membrane and probed with α -GFP or α -HA primary antibody and horseradish peroxidase coupled secondary antibody. Loading was monitored by PonceauS (PonS) staining of the membrane.

Figure S5

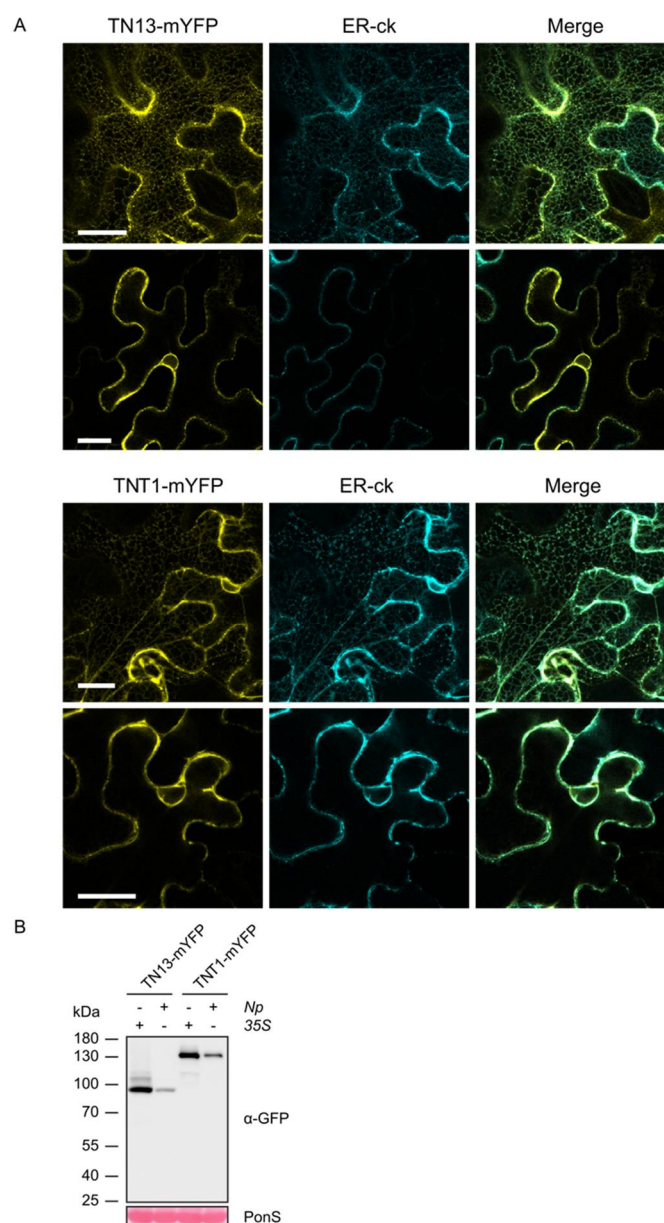


Figure S5. Localization of TN13-mYFP and TNT1-mYFP upon *Agrobacterium*-mediated transient expression in *Nbeds1a-1* leaves under control of the respective native promoters. (A) Confocal laser scanning microscopy was performed 2 days after *Agrobacterium* infiltration. The top panels show z-stacks, the panels below show a single confocal plane crossing the nucleus. TN13-mYFP and TNT1-mYFP are shown in yellow, the co-expressed CFP-tagged ER marker (ER-ck, Nelson *et al.*, 2005) is shown in cyan. Scale bar = 25 μ m. (B) Immunoblot analysis of total protein extracts derived from infiltrated *Nbeds1a-1* leaf tissues expressing TN13-mYFP and TNT1-mYFP under control of the native (*Np*) or 35S promoter. Samples were separated on a 7.5 % SDS polyacrylamide gel, blotted onto a nitrocellulose membrane and probed with α -GFP primary antibody and horseradish peroxidase coupled secondary antibody. Loading was monitored by PonceauS (PonS) staining of the membrane.

Figure S6

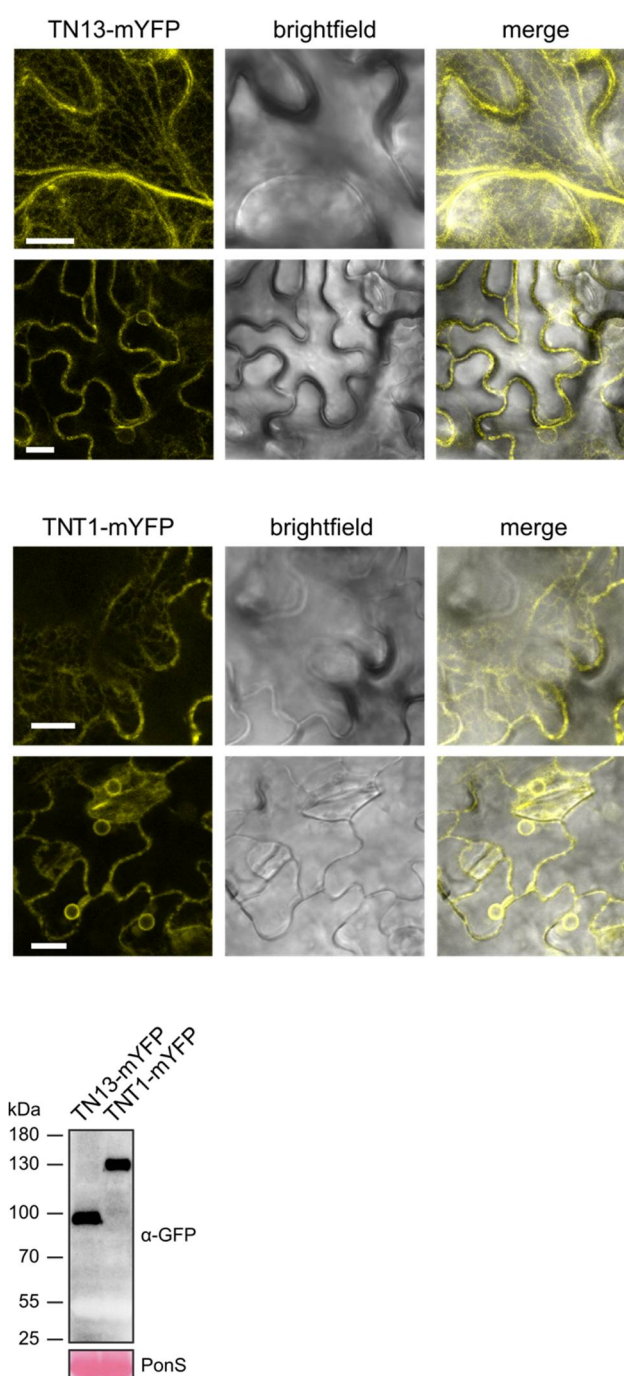


Figure S6. Localization of TN13-mYFP and TNT1-mYFP in stable transgenic *Arabidopsis* lines, expressing the fusion protein constructs under control of the 35S promoter. Line #12-3 and #4-1 have been used respectively (see Figure S2). (A) Confocal laser scanning microscopy was performed on 2 weeks old plants, grown on $\frac{1}{2}$ MS agar plates. The top panels show z-stacks, the panels below show a single confocal plane crossing nuclei. TN13-mYFP and TNT1-mYFP are shown in yellow. Scale bar = 25 μ m. (B) Immunoblot analysis of total protein extracts, derived from plants used for CLSM in (A). Samples were separated on a 7.5 % SDS polyacrylamide gel, blotted onto a nitrocellulose membrane and probed with α -GFP primary antibody and horseradish peroxidase coupled secondary antibody. Loading was monitored by PonceauS (PonS) staining of the membrane.

Figure S7

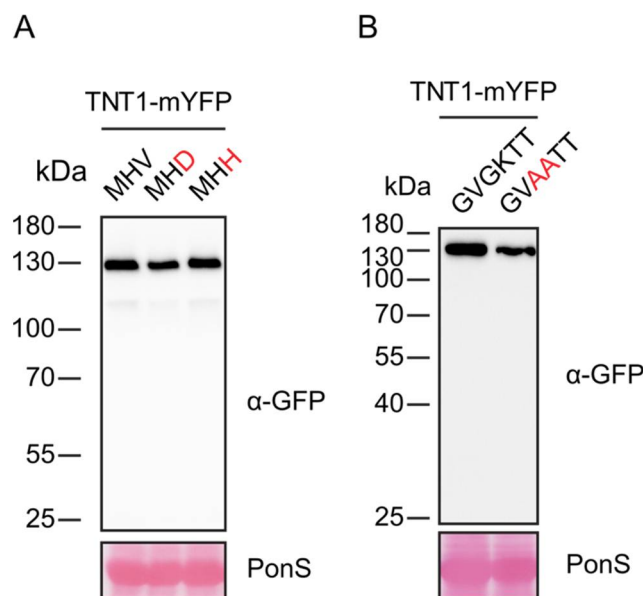


Figure S7. Immunoblot analysis of total protein extracts, derived from tissues transiently expressing TNT1-mYFP wild-type and mutant variants. Samples were taken 2 days after *Agrobacterium* infiltration of *N. benthamiana* wild-type plants for TNT1-mYFP mutant variants or *Nbeds1a-1* mutant plants for the TNT1-mYFP wild-type variant. All samples were separated on 7.5 % SDS polyacrylamide gels, blotted onto nitrocellulose membranes and probed with the indicated primary antibodies. Horseradish peroxidase coupled secondary antibodies were used for detection. PonceauS (PonS) staining of the membrane was used as a loading control.

Figure S8

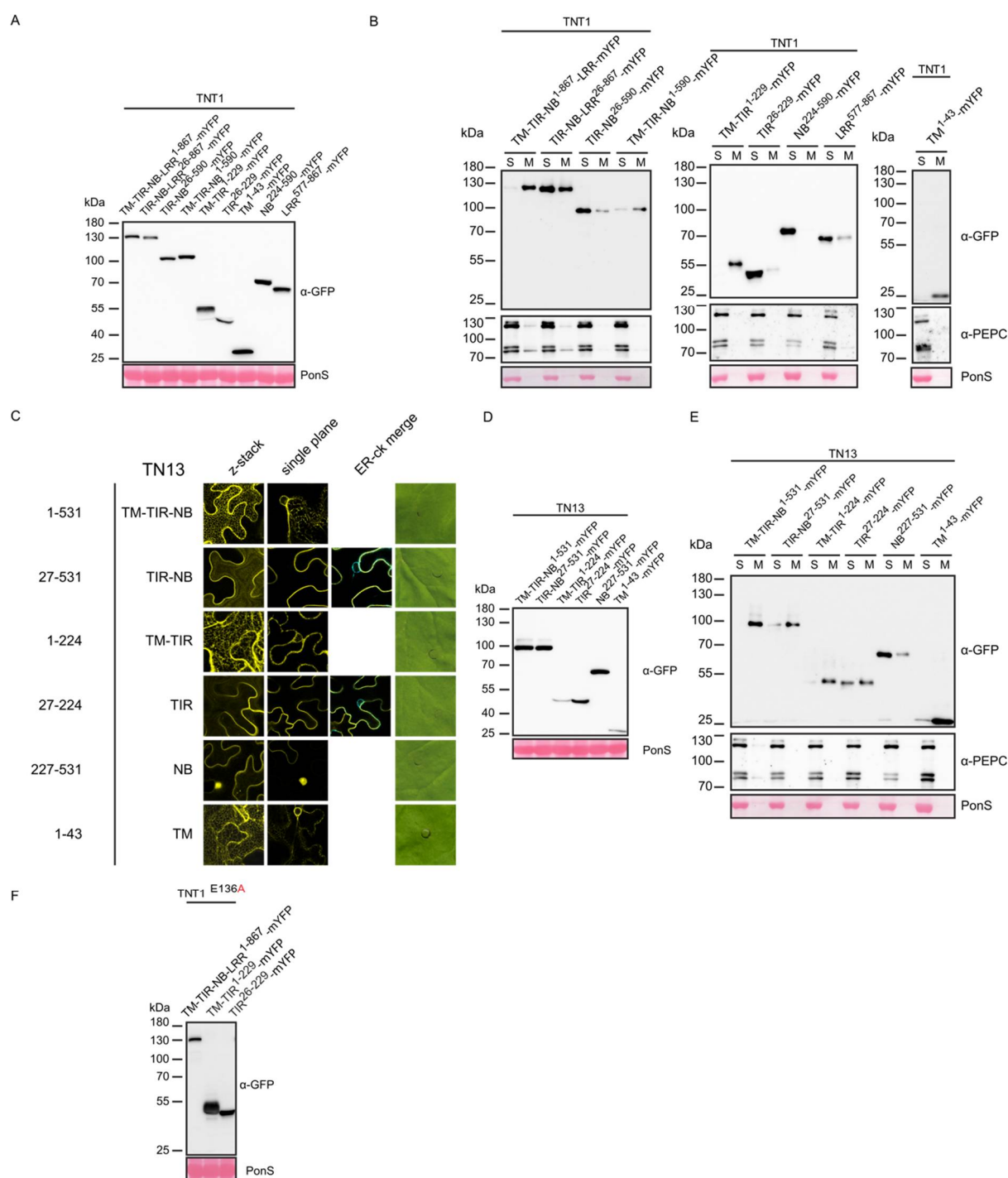


Figure S8. NLR domain truncation and mutation analysis. Immunoblot analysis of total protein extracts derived from tissues transiently expressing mYFP-tagged TNT1 wild-type and truncated versions (A), and TN13 wild-type and truncated versions (D). Samples were derived from *N. benthamiana* wild-type plants (for TN13 expression) or *Nbeds1a-1* mutant plants (for TNT1 expression) 2 days after infiltration of *Agrobacteria*. Immunoblots of protein extracts subjected to microsomal fractionation after expression of mYFP-tagged TNT1 wild-type and truncated versions (B) and TN13 wild-type and truncated versions (E). S = soluble fraction, M = membrane fraction. PEPC served as a cytoplasmic marker. (C) CLSM images of z-stacks and single confocal planes of the respective mYFP-tagged TN13 wild-type and truncated versions (shown in yellow), 2 days after infiltration of *Agrobacteria* into *N. benthamiana* wild-type plants. The ER marker fused to CFP (ER-ck, Nelson *et al.*, 2005) was co-infiltrated to mark the nuclear envelope (shown in cyan). Confocal images showing the localization of the full length TN13 protein are also displayed in Figure 3A. For cell death assays, the infiltrated leave areas were photographed 5 days after infiltration of *Agrobacteria*. (F) Immunoblot analysis of total protein extracts, derived from infiltrated tissues expressing mYFP-tagged TNT1 full length or truncated versions, containing an E₁₃₆A mutation of a predicted catalytic glutamate. Samples were taken 2 days after *Agrobacterium* infiltration of *N. benthamiana* wild-type plants. All protein samples were separated on 7.5 % SDS polyacrylamide gels, blotted onto nitrocellulose membranes and probed with α -GFP primary antibody and horseradish peroxidase coupled secondary antibody. Loading was monitored by PonceauS (PonS) staining of the membranes.

Figure S9

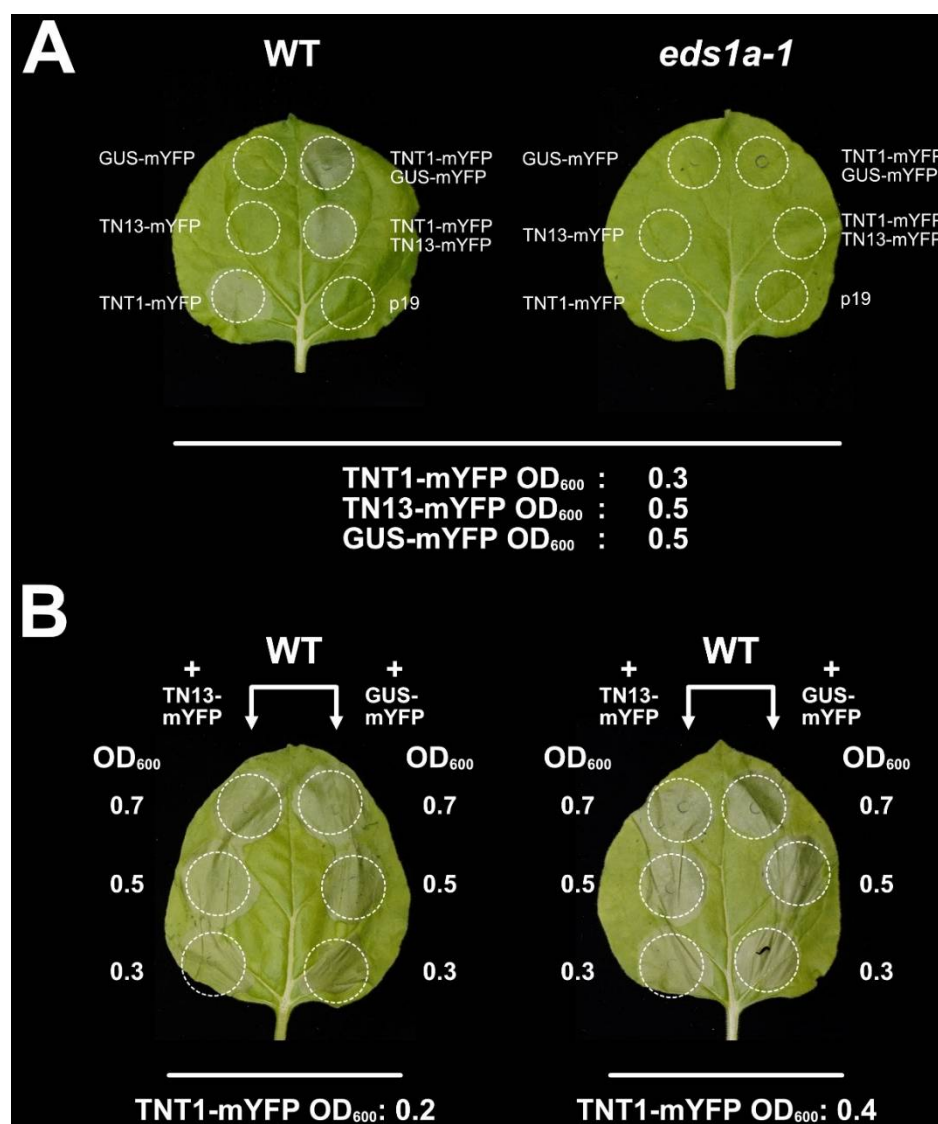


Figure S9. *Agrobacterium*-mediated transient co-expression of TNT1-mYFP and TN13-mYFP in wild-type *N. benthamiana* does not suppress TNT1 induced cell death. (A) Leaves of wild-type and *eds1a-1* *N. benthamiana* plants were photographed 4 days after infiltration of *Agrobacterium* solutions of the indicated optical densities (OD_{600}) for transient (co-)expression of mYFP tagged TN13, TNT1 or GUS under control of the 35S promoter. The TNT1-induced cell death response depends on *NbEDS1*. Expression of GUS alone and of the silencing suppressor p19 alone served as additional controls. (B) Co-expression of TNT1-mYFP with either TN13-mYFP (left side of the leaf) or GUS-mYFP (right side of the leaf) in WT *N. benthamiana* leaves under control of the 35S promoter. Leaves were photographed 4 days after infiltration of *Agrobacterium* solutions of the indicated optical densities (OD_{600}). The silencing suppressor p19 was co-expressed with all combinations.

Figure S10

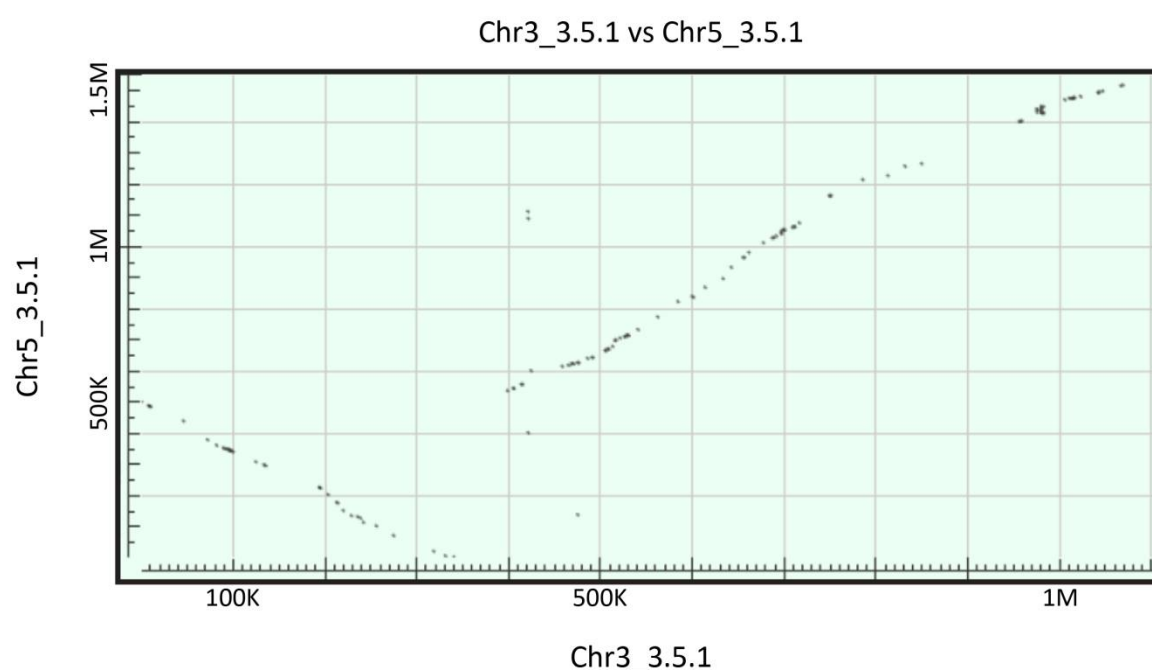


Figure S10. Dot plot matrix analysis of genomic regions. Dot matrix of alignment hits between the genomic sequences (based on TAIR10) of the regions between *AT3G01015* to *AT3G04350* on *Arabidopsis* chromosome 3 (Chr3_3.5.1), and *AT5G14060* to *AT5G18490* on chromosome 5 (Chr5_3.5.1), using the megablast settings in BLASTp. Numbers of bases are indicated.

FigureS11

```

1 .....10.....20.....30.....40.....50.....60
AT3G04220.3 1 MDSSFL-ETVAAATGFFTLGTLFMVYRKFKDH-----RENKENDSSSSSTQSPSPPL
AT5G18350.1 1 -----MQPN-----RASSSL
AT5G18370.1 1 -----MAIAEVIGFFTLVLCVIWVYVYKHKHNLQKICRSSIESSSSSSSSSSPPLS
AT3G04210.1 1 MDSYFFLGTVVVVALAIYTLGTFISFMVYRKFRHQE---KNNISSCFSFSSCFSSSS
AT5G18360.1 1 STSSPGRKHQVFPSTFHGADVRKSFSLHMKEFKSGIDIFIDK-DIKRGKSIGPELTAI
AT5G17970.1 1 -----MESSSL
AT5G17680.2 1 -----MASVSS
AT5G17880.1 1 -----MASLPSSSS
AT5G17890.1 1 -----MTSSSSSV
1 -----MEPPAARV

61 .....70.....80.....90.....100.....110.....120
AT3G04220.3 55 SLSLTKRYDVFPSTFRGEDVRKDFLSHQKEFOROGITPFVDN-NIKRGESIGPELIRAI
AT5G18350.1 12 STPTRTWTHHVFLSFRGEDVRKGFSLHQKEFERKGIFFPVDN-KMKRGSSIGPVLSDAI
AT5G18370.1 53 SPISRTWTHQVFPSTFRGEDVRKGFSLHQKEFKSGIIVPFIDD-EMKRGESIGPGLFQAI
AT3G04210.1 58 STSSPGRKHQVFPSTFHGADVRKSFSLHMKEFKSGIDIFIDK-DIKRGKSIGPELTAI
AT5G18360.1 8 SLOSCNWRHHVFPSTFGKDVRTFLSHLLKEFKRGIRTFFDN-DIKRSOMISSELVRAI
AT5G17970.1 8 SSFSDRHSYHVFPSTFGEDVRNFLSHLHKELOHNGIDAFKDG-GIKRSRSIWPELKQAI
AT5G17680.2 10 SSSSTVWKTDFVFSFRGEDVRKTFSLHCEFDRMGIKAFRDLDLQKGSISPELDAI
AT5G17880.1 9 KTDGETPDQVFINFRGVETIRKNEVSHLEKGLKRGINAFIDT-DEEMGQELSV-LLERI
AT5G17890.1 9 TPSIKADCSHSVNIICEETVLHSLSHLSAALRRREGISVFDACGLQETKFFSIKQNP

121 .....130.....140.....150.....160.....170.....180
AT3G04220.3 114 R-GSIAIILLSNYASSSWCLDELVEIKCEEMGQTVVIFYKVDPSLVKKLTGDFGK
AT5G18350.1 71 I-VSRIAIVLSNYASSSWCLNELVNIMKCEFFGQTVMTVFYEVDPDVRKQTDGFGI
AT5G18370.1 112 R-ESKIAIVLSNYASSSWCLNELVEIMKCEEIGQTVMTVFYQVDPDVRKQTDGFGK
AT3G04210.1 117 R-GSRFAIVFLSNYASSSWCLNELALIMKCKELGLTVTLFYDIDPDVRKQTDGFGM
AT5G18360.1 67 R-ESRIAIVLSNYASSSWCLNELVEIKK---VSQMIIMPVFYEVDPDVRKRTGDFGK
AT5G17970.1 67 W-ESIFIVLSNYASSSWCLDELVEIMECEVVGKTVVIFYVDPSVVRKQTDGFGK
AT5G17680.2 70 R-GSRFAIVVSNYAASSWCLDELKIMECNK---TVVIFYEVDPDVRKQRGSGFE
AT5G17880.1 67 E-GSRIAIFSPRYTESKWCLELAKKERTQKELVVIFYKVDPSLVKKLTGDFGK
AT5G17890.1 69 TDGARLVVVSDEVEFYDPWFFKFKVIGWQNNGHVVIFYGVDD----SLTRVIGW

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AT3G04220.3 173 VFRNTCKGR-ERENIEWREAFKKVATAGYDSRKWDNESGMIEKIVSDISEMLNHSTP
AT5G18350.1 130 AFETTCVGR-TEEVKQSWROALIDVSNIVGEVYRIWSKESDLIDKIAEDVLDGLNY-TM-
AT5G18370.1 171 AFKKTCVGR-TOEVKQWRSRALMDVANILGQDSRKWDKEADMIVKAKDVSVDLSY-TP-
AT3G04210.1 176 AFKETCKGR-TKEIGWRHAEVVAKIAGYHSSIWWDNEADMIGIVTTDISNTLNISTP
AT5G18360.1 122 AFEEACERQPDDEVKQWRALVYANLAGESSQWWDNEADLIDKIAMSISYELNS-TL-
AT5G17970.1 126 AFDKICDVR-TEERQWRQALTNVNIAGECSSKWDNDAKMIKIVAYVSEELFCFTS-
AT5G17680.2 126 DVESH---S-DKEKVGWWEALKKAAISGEDSRNWRDSSKLKKIVKDISDKLVS-TS-
AT5G17880.1 126 KFRELVKST-DKKTKEWWEALQYVFFITGIVLDEKSDDEVINIIIRKVKEILNRRSEG
AT5G17890.1 124 A---NSWLEAEKLTSHQSKILSNNVLTDELVEEIVRDVYVKLYP----

241 .....250.....260.....270.....280.....290.....300
AT3G04220.3 231 -----SDFDILG GDHMEK KPLLDIDSD- MKTIGIWGPPGVGKTTIAR
AT5G18350.1 187 -----SDFDGYVGIGRHRK KSLLCSSG- VRMIGIVGPPGIGKTTIAR
AT5G18370.1 228 -----SDFDGYVGIRPH TRNSLLCSSL- VRMIGILGPPGIGKTTIAR
AT3G04210.1 234 -----SSDFS LVG EDHMKKLER LYLALND- VRMIGIVGPPGIGKTTIAR
AT5G18360.1 180 -----SDSYNLVGI DNHMRELD SLLCLSTE- VRMIGIWGPAGIGKTTIAR
AT5G17970.1 184 -----STDFTLGL EAH ANLKS LGLSNE- VRMIGIWGPAGIGKTTIAR
AT5G17680.2 180 -----WDDSKGLG SSHMDFLQSTISVDKD- VRMIGIWGMGGVGKTTIAR
AT5G17880.1 185 PPSKCSALPPQRHQRHETFWGIELR KOLEEKLRFSGD TTRTIGVGMFPGIGKTTIAR
AT5G17890.1 166 -----ARVGIYAR LE EKLLYKQHRD- IRSIGIWGMFPGIGKTTIAR

301 .....310.....320.....330.....340.....350.....360
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AT5G18350.1 233 ALRDOIS---ENFOLTAFTDFTLP-RRCYGSGLKPPTAFMNDNR-RKVLQTNFL
AT5G18370.1 274 VLYDOS---KFOFAFENIFLSW-KGWHG GNLDFPVEIMTGDQRKLNLRRL
AT3G04210.1 280 VL RKHS---DSFDLSVFMET KGYTR-PGCSDEHGL-----KLHLQOQFL
AT5G18360.1 226 ALNRIS---ENFOHTIFMEN KGSST-TSELDAYGF-----QLRLQEQFL
AT5G17970.1 230 ILYNQSSSND DFOFIFMEN KGS-RKEIDGYSM-----KLHLRERFL
AT5G17680.2 226 YLYNQS---GOFQVHCFMEN KEVC-----NRYGV-----RRLOVEFL
AT5G17880.1 245 MLYEKWN---DRFLRH LRD HEAS-----EEDGL-----NYLATKFL
AT5G17890.1 208 AVNHMS---TD DASC FENFDEA-----HKEGL-----HRLKERI

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Figure S11. Protein sequence alignment of NLRs within the segmentally duplicated regions of chromosome 3 and 5. Alignment was performed using MAFFT, shading was performed using boxshade. Identical amino acids are highlighted in black and conserved amino acids in grey. The potential catalytic glutamate residue, and MHD-like motif are indicated by red boxes.

Figure S12

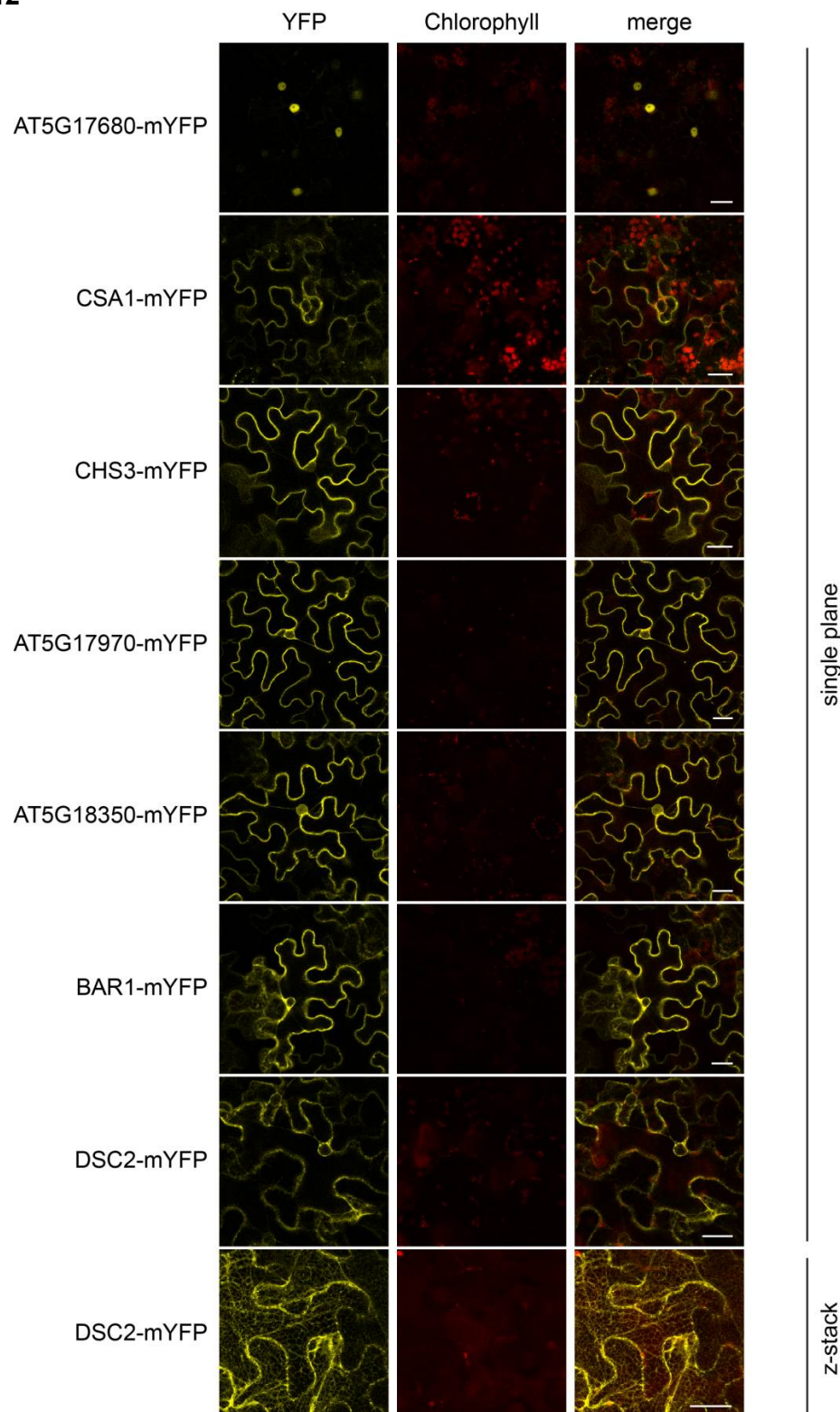


Figure S12. Localization of mYFP-tagged NLRs encoded in the segmentally duplicated cluster on chromosome 5. The localization of mYFP-tagged NLRs, encoded in the segmentally duplicated cluster on chromosome 5 was analyzed after *Agrobacterium*-mediated transient expression in *Nbeds1a-1* leaves, under control of the 35S promoter. Confocal laser scanning microscopy was performed 2 days after infiltration of *Agrobacteria*. Z-stack and single confocal planes crossing the nucleus are shown. mYFP signals are shown in yellow, chlorophyll auto-fluorescence is shown in red. Scale bar = 25 μ m.

Figure S13

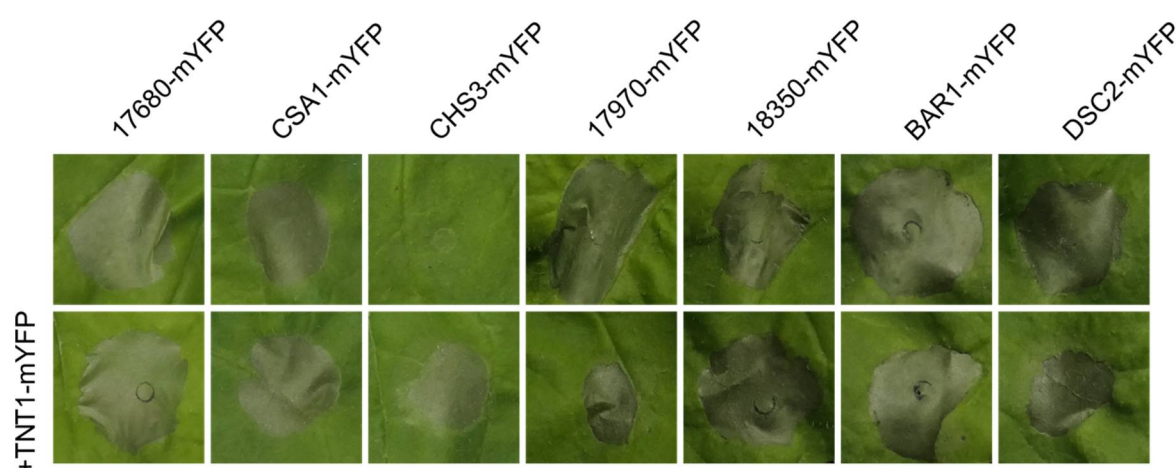


Figure S13. Cell death induction upon transient expression of NLRs encoded in the segmentally duplicated cluster on chromosome 5. *Agrobacterium*-mediated transient expression of mYFP-tagged NLRs, encoded in the segmentally duplicated cluster on chromosome 5 under control of the 35S promoter. Upper panel shows individual expression of the NLRs and lower panel shows co-expression with TNT1-mYFP. Cell death was photographed 5 days after *Agrobacterium*-infiltration.

Figure S14

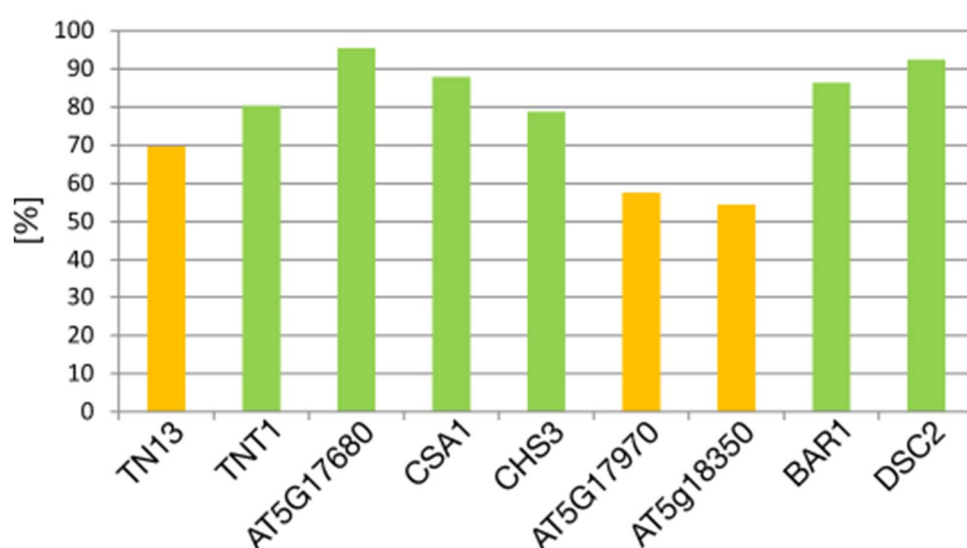


Figure S14. NLR presence analysis within *Arabidopsis* accessions. Presence (in %) of the respective orthogroups within the 65 *Arabidopsis* accessions used by Van de Weyer *et al.* (2019) to define core (>79 %, green), shell (>20 %, yellow) or cloud (<20 %, red; not applicable) NLRs within the pan-genome NLRome of *Arabidopsis thaliana*.

Table S1. Primers used in this study. The name, sequence and use of each primer is indicated. SDM = site-directed mutagenesis, GT = genotyping, RT = RT-PCR.

Name	Sequence 5'-3'	Use
TN13_Np_Fwd	CACCCCTTTGTTATTGCTTGCCG	cloning
TN13_TM_Fwd	CACCATGGATTCTTATTTTTTCC	cloning
TN13_TIR_Fwd	CACCATGGTTTATAGAAAATTCAGGTTACACC	cloning
TN13_NB_Fwd	CACCATGACGTTGAACATCTCAACGCCG	cloning
TN13_TM_Rev.Δstop	AGAAGAATCATTTTCTTTGTTTTCTCG	cloning
TN13_TIR_Rev.Δstop	AATATCAGTGGTGACTATTCCG	cloning
TN13_NB_Rev.Δstop	ATGATTCAACGACTCCGC	cloning
TNT1_Np_Fwd	CACCCTTCTTCTCCCTCTCACTTTGC	cloning
TNT1_TM_Fwd	CACCATGGATTCTTCTTTTTTACTCGAAACTG	cloning
TNT1_TIR_Fwd	CACCATGGTTTACAGAAAATTCAAAGACC	cloning
TNT1_NB_Fwd	CACCATGCTGAATCATTGACGCCG	cloning
TNT1_LRR_Fwd	CACCATGGGCAGTAGAAGTGTAATAGGCATAG	cloning
TNT1_TM_Rev.Δstop	AGATGATATGTTGTTCTTCTCTTG	cloning
TNT1_TIR_Rev.Δstop	CGTCGAATGATTGAGCATTTCG	cloning
TNT1_NB_Rev.Δstop	CGTATTGAAGTCGAAATCTATGCC	cloning
TNT1_LRR_Rev.Δstop	GCATTTATAAACTTCAATCTCTTGAG	cloning
TNT1_MHV->MHD_Fwd	CGTATGCATGaTCTGCTGGCA	SDM
TNT1_MHV->MHD_Rev	TATCAGTCTTAAATCCATATGTATAAGC	SDM
TNT1_MHV->MHH_Fwd	ACGTATGCATcaTCTGCTGGCAC	SDM
TNT1_MHV->MHH_Rev	ATCAGTCTTAAATCCATATGTATAAG	SDM
TNT1_GVGKTT->GVAATT_Fwd	CCTGGGGTTGccgcAACCACCATTG	SDM
TNT1_GVGKTT->GVAATT_Rev	AGGACCCCATATTCCTATC	SDM
TNT1_E->A_Fwd	TGCTTAGACGcATTGGTGGAG	SDM
TNT1_E->A_Rev	CCAGCTTGAAGAAGCGTAG	SDM
AT5G17680_Fwd	CACCATGGCTTCTCTTCCTTCTTCTTC	cloning
AT5G17680_Rev.Δstop	CTTTCGTATGACATTTGCTGATG	cloning
AT5G17880_Fwd	CACCATGACAAGCTCCTCCTCCTG	cloning
AT5G17880_Rev.Δstop	ATGGCTATACATTTCATAAAGCACG	cloning
AT5G17890_Fwd	CACCATGGAACCACCAGCTGCTC	cloning
AT5G17890_Rev.Δstop	TAACCTTGAATATTGTGGAGTCTTG	cloning
AT5G17970_Fwd	CACCATGGCTTCTGTTTCGTCCTC	cloning
AT5G17970_Rev.Δstop	TATGTCCACGATCTCTATACTTGACG	cloning
AT5G18350_Fwd	CACCATGCAACCTAATCGAGCCTCG	cloning
AT5G18350_Rev.Δstop	TTCTCTTTCTTGCTGGAGATTGATCC	cloning
AT5G18360_Fwd	CACCATGGAAGAATCTTCATCTTTGTCATTG	cloning
AT5G18360_Rev.Δstop	TTTATCGAACCAGATGAAATCATTCTC	cloning
AT5G18370_Fwd	CACCATGGCTATTGACAGAGGTGATTG	cloning
AT5G18370_Rev.Δstop	ATCGTCTCCATGAGCATGAGG	cloning
tnt1-1_Fwd (LP)	TTGTAAAGAAGCTGACCGGTG	RT/GT
tnt1-1_Rev (RP)	CGCCAAGGCATCTACTTGAC	RT/GT
tnt1-2_Fwd (LP)	GGCACTACCAAGGGTAAGGAC	RT/GT
tnt1-2_Rev (RP)	TTTAAACAAGGAACCTCCGCATG	GT
tn13/tnt1_Fwd_c	AGCTGGTGGATGTGAAGTGG	GT
tn13/tnt1_Fwd_b	GGATTTACCACCACCTGATGATGC	GT

tn13/tnt1_Rev_a	ACATCCACAGCCGTATCAGG	GT
tnt1-2_Rev_RT	GAGGGTAATTCCACCAGAGATAAGC	RT
TUB_Fwd	ACGTATCGATGTCTATTTCAACG	RT
TUB_Rev	ATATCGTAGAGAGCCTCATTGTCC	RT