Activation of a Plant NLR Complex through Heteromeric Association with an Autoimmune Risk Variant of Another NLR

**Highlights**

- Two unlinked plant NLRs physically interact to trigger autoimmunity
- The N-terminal TIR domains mediate heteromeric NLR association
- NLR multimerization is not sufficient for signaling

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**In Brief**

Mismatched combinations of plant immune proteins can trigger autoimmunity in hybrids. Tran, Chung et al. report that a pair of NLR immune receptor variants, encoded by unlinked loci, triggers autoimmunity through heteromeric association. Signaling activity of the NLR complex depends on the sum of activation potentials of individual partner NLRs.
Activation of a Plant NLR Complex through Heteromeric Association with an Autoimmune Risk Variant of Another NLR

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SUMMARY

When independently evolved immune receptor variants meet in hybrid plants, they can activate immune signaling in the absence of non-self recognition. Such autoimmune risk alleles have recurrently evolved at the DANGEROUS MIX2 (DM2) nucleotide-binding domain and leucine-rich repeat (NLR)-encoding locus in A. thaliana. One of these activates signaling at the presence of a particular variant encoded at another NLR locus, DM1. We show that the risk variants of DM1 and DM2d NLRs signal through the same pathway that is activated when plant NLRs recognize non-self elicitors. This requires the P loops of each protein and Toll/interleukin-1 receptor (TIR)-domain-mediated heteromeric association of DM1 and DM2d. DM1 and DM2d each resides in a multimeric complex in the absence of signaling, with the DM1 complex shifting to higher molecular weight when heteromerizing DM2 variants are present. The activation of the DM1 complex appears to be sensitive to the conformation of the heteromizing DM2 variant. Autoimmunity triggered by interaction of this NLR pair thus suggests that activity of heteromeric NLR signaling complexes depends on the sum of activation potentials of partner NLRs.

INTRODUCTION

The ability to discriminate non-self from self is essential for effective immunity. In plants and mammals, nucleotide-binding domain and leucine-rich repeat (NLR)-containing receptors have convergently evolved crucial roles in recognition of non-self and modified-self molecules as danger signals [1]. In plants, NLRs can directly associate with pathogen-derived effectors, or they can indirectly sense effector-mediated modification of other host proteins. Some of these are so-called NLR guaidees, proteins that are targeted by effectors because their suppression enhances virulence, whereas others are decoys that merely entrap effectors and that have no direct function in host resistance [2, 3]. Upon recognition, plant NLRs are thought to undergo nucleotide-dependent conformation changes, most likely by relieving autoinhibitory intramolecular contacts[4]. This in turn exposes the N-terminal coiled-coil (CC) or Toll/interleukin-1 receptor (TIR) domain for participation in higher-order signaling complexes [5–10].

NLR domains that directly engage pathogen effectors, such as the leucine-rich repeat (LRR) domain, are highly diverse, reflecting effector-NLR co-evolution [11, 12]. Binding specificity for effector variants translates into a matching recognition spectrum of NLRs, with contributions mainly, but not exclusively, from LRR domains [12–15]. Extensive structure-function studies have shown that the different domains have to be finely matched for optimal specificity and robustness of NLR signaling. In agreement, minor changes can drastically alter the activity and specificity of NLR variants [16–18], and swapping even a small domain between variants can trigger severe autoimmunity [19, 20].

One way to increase specificity without compromising the strength of an immune response is to partition recognition and signaling functions into distinct NLRs. In several examples of such pairs, one partner signals whereas the other no longer needs a functional P loop, but has an extra domain that directly interacts with effectors [21–24]. Pairs functionally characterized to date are encoded by adjacent genes at the same genomic locus, but a few NLR pairs encoded by unlinked loci function in a similar manner. In mammals, NAIP and NLRC4 NLRs serve as co-receptors, with different NAIP isoforms sensing distinct, conserved pathogen molecules and NLRC4 mainly transducing signals [25, 26]. In plants, several NLRs, such as tobacco NRG1, Solanaceae NRC1-like proteins, and A. thaliana ADR1 proteins, mediate signaling from unlinked, effector-sensing NLRs [27–30], although it is not known whether these NLRs reside in the same complex.

Given the demands placed on an immune system that has to detect and rapidly respond to a multitude of pathogens, it may not come as a surprise that things can go wrong, and sometimes very badly so. This is the case in some hybrid plants, where independently evolved immune system components are mismatched...
and trigger inappropriate immune reactions in the absence of pathogens. Because cell death is a typical symptom, this syndrome is known as hybrid necrosis [31]. Hybrid necrosis is often caused by interacting alleles at two unlinked loci, and alleles from complex NLR loci are the most common cause [32–35]. An example where both interacting alleles encode NLR variants is provided by the unlinked TIR-NLR (TNL) loci DANGEROUS MIX1 (DM1) from A. thaliana accession Uk-3 and DM2 from Uk-1. Both accessions were collected in Umkirch, Germany [32, 36]. DM2, which is part of the RPP1 supercluster involved in direct recognition of ATR1 effectors [15], recurrently generates hybrid necrosis risk alleles that can also interact with non-NLR alleles [34, 36].

We demonstrate that the autoimmune risk DM1Uk-3 and DM2dUk-1 (hereafter DM1 and DM2d) NLRs associate and form an autoimmune signaling complex in planta. Because the association between DM1 and DM2g, a close paralog of DM2d, also from Uk-1, fails to trigger signaling, we propose that signaling activity depends on the conformation of the heteromerizing NLR to a DM1 oligomer complex. This autoimmune heteromeric NLR complex, which does not require an effector trigger, provides a unique experimental paradigm for investigating NLR signaling.

RESULTS

Genetic Requirements for DM1/DM2d-Triggered Autoimmunity

To better understand how DM1/DM2d-triggered autoimmunity relates to NLR/effector-stimulated immune responses, we examined the effects of mutations in several regulators of NLR-mediated cell death. EDS1 can act by direct association with TNLs [37, 38], whereas an HSP90-, SGT1-, and RAR1-containing chaperone complex has several roles in immunity, including regulation of NLR protein stability [39]. The NADPH oxidase RBOHD modulates the spread of NLR-induced cell death through its role in generating reactive oxygen species (ROS) [40, 41]. To test the effects of mutations in EDS1, SGT1B, RAR1, and RBOHD, we generated wild-type or mutant plants (Table S1) that were homozygous for gDM1-2xHA (gDM1-HA) or gDM2d-4xMyc (gDM2d-Myc) transgenes. We combined the two transgenes in each of the five backgrounds through crosses. The F1 progeny were grown at 16°C, a temperature at which hybrid necrosis is strongly expressed. The control F1 hybrid plants were very small and their leaves had many necrotic spots, similar to F1 hybrids of the Uk-1 and Uk-3 accessions, the donors of the DM1 and DM2d genes (Figures 1A and

Figure 1. Genetic Requirement for DM1/DM2d-Dependent Autoimmunity (A) Rosettes of 30-day-old A. thaliana plants expressing gDM1-HA, gDM2d-Myc, or both (F1) in the indicated backgrounds. The numbers indicate the proportion of the presented phenotype in three sowings. Scale bars, 1 cm. (B) Expression analysis of immunity marker genes PR1, NPR1, and WRKY46. Relative expression of each marker gene in F1 hybrids is indicated as −(ΔΔCT) values from three biological replicates (different color dots), with three technical replicates (one dot for each data point). Tukey’s multiple comparison test was used to test for statistical significance (alpha = 0.05). ***p < 0.0001, **p < 0.001, *p < 0.01, ns, p > 0.05, non-significant. See also Figure S1 and Table S1.
TT motif in DM1 and TT (Figures 2A and 2B). To quantify DM1/DM2d N. benthamiana (Figures 2A and 2B)—were epitope tagged and expressed in partial NB, TIR-NB and extended ARC, and LRR domains (Figure S1C). DM1/DM2d fragments. Four fragments each—the TIR, TIR plus partial NB, TIR-NB and extended ARC, and LRR domains (Figures 2A and 2B)—were epitope tagged and expressed in N. benthamiana, with β-glucuronidase (GUS) expression as negative control. The combination of full-length DM1-HA with DM2d-Myc caused confluent cell death 4 days post-infiltration (dpi) (Figure S1C), similar to the non-epitope-tagged proteins DM1-HA and DM2d-Myc—from control plants eventually set seeds, despite reduced growth and tissue necrosis (Figure S1B).

DM1/DM2d signaling thus requires factors used by other TNLs after pathogen recognition, such as EDS1 and the HSP90/SGT1B/RAR1 chaperone complex. DM1/DM2d signaling, however, does not require RBOHD-dependent ROS bursts, a result reminiscent of the lesion simulating disease 1 (lsd1) mutant, whose phenotype depends on the atypical NLR ADR1-L2 [40].

Requirement of Full-Length Proteins with Functional P Loops

A convenient platform for testing NLR activities is transient expression in Nicotiana benthamiana. Co-expression of full-length DM1 and DM2d can induce a robust, pathogen-independent hypersensitive response (HR) in this system [36]. Because N-terminal fragments of several plant TNLs can trigger HR on their own in N. benthamiana [42], we also tested DM1 and DM2d fragments. Four fragments each—the TIR, TIR plus partial NB, TIR-NB and extended ARC, and LRR domains (Figures 2A and 2B)—were epitope tagged and expressed in N. benthamiana, with β-glucuronidase (GUS) expression as negative control. The combination of full-length DM1-HA with DM2d-Myc caused confluent cell death 4 days post-infiltration (dpi) (Figure S1C), similar to the non-epitope-tagged proteins [36]. Unlike the combination of DM1 and DM2d full-length proteins, none of the fragments on their own were sufficient to trigger HR, despite robust protein accumulation (Figures S1D and S1E). We did not observe any HR either when combinations of the DM1 and DM2d fragments were co-expressed (Figure S1C). The requirement of full-length paired proteins thus differentiates DM1/DM2d-dependent signaling from several unusual NLRs, whose N-terminal domains alone are sufficient to trigger full or partial HR [5, 7, 9, 10, 15, 42].

P loop mutations render many NLRs inactive [4]. In addition, NLR activity is altered by mutations of the MHD motif, presumably because such mutations change local conformation around the ATP-binding pocket [43, 44]. In particular, a change from MHD to MHV causes several NLRs to preferentially bind ATP, and thereby makes them constitutively active [43, 45].

To investigate whether DM1/DM2d signaling depends on P loop activity, we mutated the GIGKKT motif in DM1 and DM2d to GIAYTT (Figures 2A and 2B). To quantify DM1/DM2d signaling, we established an ethanol-inducible system for DM1-HA expression (indDM1-HA) in N. benthamiana (see the Supplemental Experimental Procedures), with inducible GFP as a control. As a proxy for cell death, we measured ion leakage. We observed confluent HR 3 days after ethanol induction of DM1-HA when DM2d-Myc was co-expressed from its native promoter (Figures 2A and 2B), validating the system. We began to measure ion leakage before cell death became apparent, and stopped measurements when ion leakage plateaued after about 22 hr (Figures 2C and 2D). When DM1 or DM2d P loop mutants were co-expressed with their wild-type partner, HR was abolished (Figures 2A and 2B). Ion-leakage kinetics with the mutant versions were similar to controls (Figures 2C and 2D).

Turning to the MHD-like motifs (MHH in DM1 and MHT in DM2d), we found that, different from other systems [43, 45], neither MHV variant was constitutively active on its own (Figures 2A–2D), and both MHV variants behaved comparable to their wild-type counterparts when co-expressed with the partner NLR (Figures 2C and 2D). Furthermore, the MHD-to-MHV substitution could not overcome the loss of activity due to P loop mutations (Figures 2C and 2D). Protein blots confirmed that mutant proteins were expressed at levels comparable to wild-type versions (Figures 2E and 2F). We conclude that DM1/DM2d signaling requires the P loops of both NLRs. In addition, neither single NLR can provide full activity, because both are resistant to MHV mutations, which render other NLRs constitutively active, presumably by shifting the ratio of ATP and ADP bound at the P loop [18].

Homotypic DM1 Association and Heterotypic DM1/DM2d Association

Signaling from some plant NLRs involves homo- or heteromerization, with the N-terminal TIR or CC domains providing dimerization interfaces [5–7, 9, 10, 21, 22]. To examine DM1/DM2d protein interactions, we began with yeast-two hybrid (Y2H) assays, often used to study plant NLRs [22]. A DM1 fragment that included the TIR and partial NB domain (TIR-pNB) interacted both with full-length DM1 and with three TIR-containing DM1 fragments (Figures 3A). DM1 TIR-pNB also interacted, albeit more weakly, with DM2d TIR and TIR-pNB fragments (Figure 3A). Neither the LRR fragments of DM1 and DM2d nor full-length DM2d showed evidence of interaction, possibly due to insufficient protein accumulation (Figures 3A and S2A). We did not detect DM2d self-association in yeast, also most likely because of insufficient protein accumulation (Figures S2B and S2C). We conclude that the N termini of DM1 and DM2d provide an interface for homo- and/or heterotypic interactions.

We confirmed the Y2H results with transient co-expression of gDM1-Myc and either gDM1-HA or gDM2d-HA in N. benthamiana (Figures 3B and 3C). Co-immunoprecipitation demonstrated that DM1-Myc could associate with both DM1-HA and DM2d-HA (Figure 3C, lanes 5 and 6). DM1-HA was much more efficiently co-immunoprecipitated than DM2d-HA, even though DM2d-HA was expressed at higher levels (Figure 3C, compare inputs). The difference may reflect a different stoichiometry of DM1 and DM2d in the signaling complex or differences in the binding affinity between DM proteins, but it might also have a more trivial explanation, with DM1/DM2d complexes being depleted because of rapid death of cells containing both proteins. Nonetheless, our data indicate strong DM1 self-association, and DM1/DM2d association. We also detected DM2d-HA/DM2d-Myc complexes (Figure 3C, lane 7), indicating that, like DM1, DM2d can self-associate.

We further confirmed the interaction between DM1 and DM2d using an A. thaliana F1 progeny of lines that expressed...
The expression of DM1-HA was induced with ethanol in 15-day-old seedlings grown at 23°C. Whereas non-induced F1 and parents carrying the individual transgenes did not show HR symptoms, ethanol-treated individuals from six independent F1 lines became necrotic (Figure 3D, red arrowheads). When we analyzed leaf samples from the treated F1 individuals in bulk, we could readily detect DM2d-Myc after pull-down with DM1-HA (Figure 3E). This result is...
consistent with both Y2H and *N. benthamiana* co-immunoprecipitation data.

We used blue native-PAGE to investigate the composition of DM1 and DM2d complexes in *A. thaliana*. Both DM1-HA and DM1-Myc proteins were found in a complex of approximately 500 kDa, whereas DM2d proteins were in an even larger complex (Figure 3F). In extracts from plants expressing both proteins, DM1-HA and DM2d-Myc co-migrated, both being detected in a complex that was larger than what we saw for DM1 alone (Figure 3F). A similar increase in complex size was observed when plants expressed both DM1-HA and DM2g-Myc (Figure 3F), a combination that does not trigger autoimmunity [36]. These results suggest that DM2 molecules may alter existing DM1 complexes through heterotypic interactions but that only the DM2d variant forms a heteromeric complex with DM1 that triggers autoimmunity.

**DM1/DM2d Association and Signaling**

Crystal structures of RPS4 and RRS1 NLRs have revealed two important residues, Ser and His (SH motif), at the hetero- and homodimeric interfaces of their TIR domains [22]. We therefore tested whether the SH motifs of DM1 and DM2d are critical for their physical association. In Y2H assays, SH mutations compromised both homo- and heterotypic interactions (Figures 4A and S3A), suggesting that the DM1 and DM2d TIR domains make contacts similar to those of RPS4 and RRS1 [22]. The SH motif in DM2d was essential for HR in *N. benthamiana* (Figures 4B, S3B, and S3C), a combination that does not trigger autoimmunity [36]. These results suggest that DM2 molecules may alter existing DM1 complexes through heterotypic interactions but that only the DM2d variant forms a heteromeric complex with DM1 that triggers autoimmunity.
interactions (Figures 4C, S3D, and S3E) and HR in N. benthamiana (Figures 4D and S3F). In DM2d, both T66A and T66G mutations affected the strength of heteromeric interaction with DM1 in the Y2H assay (Figures 4E and S3G), with T66G eliminating, and T66A reducing, HR in N. benthamiana (Figures 4F and S3H).

Our experiments with variants that have mutations in or near the N-terminal SH motif in the TIR domains thus demonstrate that physical interactions at the TIR interface between DM1 and DM2 correlate with autoimmune signaling and that the stability of the interface appears to quantitatively affect signaling output.

**DM1/DM2g Association and Lack of Signaling**

Because the interaction interfaces of TNLs generally are in the TIR domain, we asked whether DM2d homologs with highly similar TIR domains can associate with DM1. The closest paralog of DM2d in the same genome is DM2g, with 95% amino acid similarity in the TIR domain. Similar to DM2d, DM2g weakly associates with DM1 in a Y2H assay (Figure 5A), apparently also through the N terminus (Figure S4A). The DM1 TIR-pNB fragment can similarly associate with the TIR-pNB fragment of RPP1 WsA, although not of other DM2h-type RPP1 homologs (Figures 5B and S4B), suggesting that the DM1 TIR domain can interact with TIR domains from a certain range of DM2/RPP1 proteins (Figure S5A). To confirm that failure of the DM2g/DM1 combination to trigger immune signaling [36] is not due to insufficient DM2g accumulation, we expressed DM2g not only from the DM2g or DM2d promoters but also from the cauliflower mosaic virus 35S promoter. None triggered HR in N. benthamiana in combination with DM1 (Figure 5C), even when protein levels were high (Figure 5D). We conclude that physical association of DM2 paralogs with DM1 is insufficient for immune signaling.

To further define the functional domains determining DM2d activity, we tested a series of DM2d promoter-driven DM2d/DM2g chimeras in N. benthamiana. Differences between DM2d and DM2g reside mostly in the TIR and LRR domains and the extended C-terminal region (Figure 5E, top panel). Replacement of either the TIR or LRR domain rendered DM2d inactive (Figure S4C, TIR swap 1 and LRR swap 2), whereas introducing either the DM2d TIR or LRR domain into DM2g was insufficient to trigger HR in combination with DM1 (Figure S4C, TIR swap 2 and LRR swap 1). Replacing the DM2d NB-ARC domain with that from DM2g did not inactivate DM2d (NB-ARC swap 1), whereas the reverse chimera (NB-ARC swap 2) was inactive (Figures S4C and S4E). The swapping results thus suggest that the TIR and LRR domains together determine DM2d-like properties.

To further narrow down the LRR region that functions in concert with the TIR domain in DM2d, we used the DM2d chimera with the NB-ARCDM2g domain (Figure 5E, NB-ARC swap 1) as a backbone and generated additional chimeras in which DM2d LRR segments were successively replaced by ones from...
Figure 5. Association of DM1/DM2 Variants and Domains Determining DM2d Activity

(A) Y2H assays of the DM2g 1–359 TIR-pNB fragment. Similar to the homologous DM2d 1–358 fragment, it associates with DM1 1–308.

(B) Y2H assays of DM2h and RPP1 TIR-pNB fragments. RPP1 WsA, but not DM2h-type, variants interact with DM1 1–308. Protein blots show that all of the fragments were expressed in yeast. Note that DM2h-type variants have an extended N-terminal region before the TIR domain (N-TIR) similar to RPP1 WsA and NdA variants (Figure S5A) [7].

(C) Lack of HR in N. benthamiana by co-expression of DM1WT and DM2g under different promoters (4 dpi). Scale bar, 1 cm.

(legend continued on next page)
DM2g (Figure 5E, LRR swaps 3, 4, and 5). The results with the first three chimeras pointed to LRRs 5–8 being important (Figures 5E and 5F). Additional chimeras showed, however, that these LRRs were not sufficient for activation (Figures S4D and S4F, LRR swaps 6, 7, and 8), indicating that differences in the second half of the LRR domain and the extended C-terminal region, which include several indels (Figure S5B), are critical. Together, we conclude that polymorphisms in the TIR domain, the second half of the LRR domain, and the extended C-terminal end of DM2d confer the ability to trigger autoimmunity through DM1, presumably by affecting intramolecular inhibitory interactions [7].

Unequal Contributions of DM1 and DM2d to Signaling

DM1 and DM2d and their paralogs have variant MHD motifs, MHH or MHT (Figure 6A). Non-canonical MHD-like motifs are also found in other plant NLRs, particularly in NLRs whose activity does not depend on a functional P loop, such as RRS1 and orientation of the K260 amino tail in the P loop (Figure 6D) and that T541H changed the orientation of the histidine in the MHD-like motif (Figure S6I). The DM1 variants tested were not predicted to greatly alter arrangement of these residues (Figures 6C, S6G, and S6H). Given that both DM2d and DM2g exist in a heteromeric complex with DM1 but only DM1/DM2d is active (Figure 3F), we propose that the MHD-like motif, in concert with other polymorphisms (Figure 5E), confers a conformation on DM2d such that it is poised to trigger signaling upon interaction with DM1.

To further investigate the relative contribution of DM1 and DM2d to signaling, we designed a competition assay in which either partner is replaced with an increasing quantity of its inactive counterpart. We used transient expression in N. benthamiana, adding an Agrobacterium tumefaciens inoculum for the competitor at three different OD600s (0.525, 1.05, and 2.1) to constant DM1 WT and DM2d WT inocula (both at an OD600 of 0.525 before mixing).

(D) Protein blots for experiments shown in (C), with samples taken at 2 dpi.
(E) Domain swaps between DM2d and DM2g. Purple vertical lines indicate SNPs, and orange bars indicate indels. Green bars indicate DM2g fragments. The numbers refer to positions in DM2d. LRRs are numbered according to [36]. N. benthamiana leaves are shown on the right (4 dpi).
(F) Protein blots for experiments shown in (E), with samples taken at 2 dpi. Ponceau-S staining is shown to indicate loading (B, D, and F).

See also Figures S4 and S5.
We first validated the competition assay system with DM1WT as competitor. Excess DM1WT did not change HR greatly (Figures 7A–7C and S7A), indicating that neither the increased inoculum nor the altered DM1:DM2d ratio affected our assay. We then tested as competitors two inactive DM1 variants, the P loop mutant DM1G223A K224A (Figure 2C) and an inactive chimera carrying the LRR domain from innocuous At3g1750, the DM1 homolog from Col-0 (DM1(G495–988)) (Figures S7B and S7C). Increasing amounts of either competitor reduced the HR extent, with DM1G223A K224A being a more effective competitor than DM1(G495–988) (Figures 7D, 7F, and 7H). Quantitative reduction in signaling by successive replacement of DM1WT with the inactive DM1 variants points to a critical signaling role of DM1 in the DM1/DM2 interaction.

Different from DM1, increasing amounts of DM2d competitors, the P loop mutant DM2dG259A K260A (Figure 2D) or the close paralog DM2g (Figures 5C and S4B), were much less efficient in suppressing HR (Figures 7E, 7G, and 7I). Our interpretation of the limited effects of DM2d competitors is that small amounts of DM2dWT are sufficient to trigger immune signaling. We confirmed that the P loop mutants of DM1 and DM2d retained their interaction properties in Y2H assays, at least for the N-terminal interfaces (Figures S7D and S7E), arguing against changes in interaction properties being responsible for the failure of DM2dG259A K260A to compete with DM2dWT.

**DISCUSSION**

We have described how two plant NLRs, DM1 and DM2d, interact to cause autoimmune immunity. The picture that emerges is that DM1 is the primary signal transducer, whereas DM2d triggers activation of a DM1 complex via heteromerization. Evidence for an unequal contribution of the two NLRs to signaling includes different effectiveness in competition assays and differential sensitivity to mutations that most likely affect protein conformation. Several characteristics differentiate the DM1/DM2d interaction from other heteromerizing plant NLRs. First, both DM1 and DM2d are required for signaling, which sets them apart from RGA4/RGA5 and RPS4/RRS1. In these cases, one protein alone, either full-length RGA4 or the RPS4 TIR domain, can trigger HR in *N. benthamiana*. Co-expression of the partner, either full-length RGA5 or the RRS1 TIR domain, suppresses autoimmunity [21, 22]. Second, the P loops need to be active in both DM1 and DM2d. In contrast, in the other two pairs, the P loop of the effector-binding NLR, RGA5 or RRS1, is dispensable [21, 22]. Third, the DM1 and DM2d loci are unlinked in the genome, whereas RGA4/RGA5 and RPS4/RRS1 belong to a group of paired, divergently transcribed loci.

The DM1 and DM2d NLRs each forms a higher-order complex in the absence of signaling, with addition of DM2d altering the DM1 complex. We speculate that a pre-existing NLR complex may provide a scaffold for a particularly rapid immune response. In the mammalian inflammasome, the NLR NLRC4 complex acts as a scaffold. Although NLRC4 does not seem to have sensor function, gain-of-function mutations in an autoinhibitory helical domain can cause autoinflammatory disease [50]. In support of a signaling role of DM1, no cognate effector has so far been identified for *A. thaliana* DM1 homologs, but a gain-of-function mutation in a close paralog, SS4, triggers autoimmunity [51]. A plausible hypothesis is that the DM1 complex transduces signals from other NLRs, presumably sensing non-self-triggered changes in the cell, via its affinity for TIR domains of other NLRs. We have demonstrated that the DM1 TIR domain can associate with the TIR domains of closely related DM2/RPP1 variants DM2d, DM2g, and RPP1 WsA but not with the N-TIR domains (Figure S5A) of two other RPP1 variants, DM2hCol-0 and DM2hBla-1. This suggests the exciting possibility that DM1 supports signaling of other NLRs during pathogen-triggered immunity and that interaction properties of DM1 TIR determine which other TIRs can be signaling clients. Because Illumina read mapping indicates that DM1-like homologs are ubiquitous among *A. thaliana* accessions [52], it will be interesting to learn whether loss of DM1 or a dominant-negative DM1 variant affects effector triggered immunity (ETI) mediated by RPP1 variants with known cognate effectors.

The association of DM2 variants with DM1 suggests that NLR-NLR interactions occur more often than initially thought [53], presumably in an equilibrium status. Activation of the preformed complex would not merely depend on association with another NLR but on the activation potential of the other NLR, as shown by domain-swapping experiments using active DM2d and inactive DM2g. A major function of DM1 would thus be to provide a quantitative readout for conformation of another NLR, a concept similar to the proposed “helper” function of other plant NLRs [27–30]. Because in contrast to DM1, known helper NLRs do not require active P loops, we propose to classify DM1 as a “transducer” NLR.

How could a DM1 complex accommodate DM2d, with the TIR domain mediating both DM1-DM1 and DM1-DM2d association? We do not think that activation involves simple one-on-one competition for the same interaction interface. The DM1/DM2d complex might resemble the active NAI2/PLR4 inflammasome, in which the sensor NAI2 and scaffolding NLR4 are found in very different proportions [54]. NLRC4 apparently uses two different interfaces, one to bind to NAI2 and the other to recruit additional NLRC4 molecules in a successive manner.

**Figure 7. Suppression of DM1/DM2 Signaling by Inactive Competitors**

(A) Semiquantitative scoring for HR in *N. benthamiana* for competition assays. Percentages indicate the fraction of HR in the field of view. (B) Ion leakage as an indication of HR with a wild-type or P loop mutant DM1 competitor at 43 and 46.5 hpi. Boxes signify the upper and lower quartiles, and the median is represented by a horizontal black line within each box (n = 6).

(C–G) Competition assay with wild-type DM1 as a negative control (C), P loop mutant DM1G223A K224A (D), P loop mutant DM2dG259A K260A (E), inactive DM1 chimera DM1(G495–988) (F), or DM2g (G). Three different amounts of each competitor (OD600 = 0.525, 1.05, or 2.1) were mixed with constant amounts of wild-type DM1 and DM2d (both at OD600 = 0.525 before mixing). HR was scored at 4 dpi.

(H and I) Protein blots for competition assays with the highest amount of competitor (OD600 = 2.1) of DM1 (H) and of DM2d (I). Samples were taken at 40 hpi. Blots indicate no sign of co-suppression of the seed inoculum of DM1 and DM2d. Ponceau-S staining is shown to indicate loading. See also **Figure S7**.
Our competition assays suggest that a small amount of DM2d can trigger signaling, implying uneven stoichiometry of DM1 and DM2d in the DM1/DM2d signaling complex. Different from NAP1/NLRC4, DM1 presumably preforms a higher-order complex, possibly a tetramer (Figure S5F). Its signaling activity is switched on upon association with DM2d, but not with DM2g. Signaling could be initiated either by entry of DM2d into the complex, leading to recruitment of additional DM1 molecules to the complex, or by heteromeric DM2d association, filling a “gap” to complete complex formation. We also cannot exclude the possibility of de novo heteromeric complex formation. In either scenario, heteromeric interaction between DM1 and DM2d greatly facilitates full activation of the complex.

DM2d is encoded by a superlocus encoding multiple RPP1 homologs for which direct and specific association with cognate effectors has been demonstrated [14, 15]. Although RPP1 clearly has a “sensor” function and effector-triggered TIR self-association is required for signaling [7], it is not clear whether RPP1 signals alone or in combination with another NLR. Extensive analyses of the effector-binding NLRs RPP1 and L6 have suggested that the sensor domain co-evolves with other domains to generate multiple variants, each of which can occupy a distinct position in a gradual transition toward full NLR activity [13, 14]. Bernoux and colleagues have shown for the L6 and L7 NLRs that their different activation potentials correlate with ATP-dependent equilibrium status, which can be independent of effector binding [18]. An attractive hypothesis is that DM2d mimics a conformation that other DM2 variants only adopt upon effector recognition, with its peculiar conformation endowing DM2d with the ability to autonomously trigger immune responses through the DM1 complex. This hypothesis is supported by the finding that mutations predicted to change DM2d conformation (Figure S6) reduce signaling, whereas DM1 is much less sensitive to similar mutations (Figure 6). We also interpret the inactivity of the DM2d P loop mutant (Figure 2B) as being primarily due to conformational changes. Another DM2 allele, DM2h[er], an ortholog of the DM2h[er]1 hybrid necrosis risk allele [36], triggers EDS1-dependent autoimmunity in combination with different second-site changes in the genome [55]. The extended C-terminal end makes a critical contribution to DM2h[er] function, similar to what we found for DM2d (Figures 5E and S5B). If indeed the overall conformation is a major determinant of DM2-mediated autoimmune activity, either in combination with DM1 or with other molecules, this would support our assertion that specific DM2 variants, and by extension perhaps other members of the RPP1 locus, function as sensitive signaling switches.

The scenario of DM1 as transducer and DM2d as trigger does not imply that the two NLRs only function with each other. Instead, DM1 might be considered a facultative guard of sensor NLRs that easily change their conformation. If many NLRs utilize a conserved interaction interface [23], a preformed NLR complex would present a particularly versatile element in the immune response. Although many NLRs are most likely under diversifying selective pressure, the TIR and CC signaling domains are rather conserved and may provide a preformed hub for signal transduction. There are limited examples of interacting NLR pairs, and so far the field has focused on those that co-evolve because they are genetically tightly linked [56].

In summary, our work has shown that the study of hybrid necrosis can reveal new aspects of immune signaling in plants. An important question for the future is whether DM1, compared to its paralogs, has special properties that extend beyond differential interaction with DM2 paralogs and, if yes, what evolutionary forces are responsible for them.

**EXPERIMENTAL PROCEDURES**

**Plant Material**

Plants were grown in long days (16 hr light/8 hr dark). Table S1 lists stocks used for transformation with DM1 and DM2d constructs. To induce the expression of indDM1/H4 in 15-day-old A. thaliana plants, pots were irrigated with 1% ethanol and covered with a plastic dome for 72 hr. The same construct infiltrated in N. benthamiana was induced at 18 hr post-infiltration (hpi) by irrigation with 1% ethanol for 18 hr.

**Transient Expression in N. benthamiana**

A. tumefaciens was grown to an OD$_{595}$ of 1.2–1.8, and then incubated in induction medium (10 mM MES [pH 5.6], 10 mM MgCl$_2$, and 150 µM acetosyringone). The cell suspensions were normalized to an OD$_{595}$ of 0.35, and mixed 1:1 for co-infiltration into the abaxial side of N. benthamiana leaves. For competition assays, competitor DM2b was adjusted to 0.525, 1.05, and 2.1; OD$_{595}$ for wild-type DM1 or DM2d was 0.525. HR was quantified by measuring ion leakage using an Orion Star conductivity meter (Thermo Scientific) in eight replicates.

**Yeast Two-Hybrid Assay**

See the Supplemental Experimental Procedures for details.

**Protein Extraction and Co-immunoprecipitation Assay**

For detection of DM1 and DM2 proteins in N. benthamiana and A. thaliana, microbial fractions of the samples were prepared. Co-immunoprecipitation assays were performed using total protein extract from 500 mg of N. benthamiana or 1 g of A. thaliana leaf tissue. See the Supplemental Experimental Procedures for details.

**Blue Native-PAGE**

0.5 g of fresh leaf samples from 2-week-old seedlings was collected and ground in solubilization buffer (30 mM HEPES-KOH [pH 7.5], 150 mM potassium acetate, 10% [v/v] glycerol, 0.5% n-dodecyl β-maltoside, and 1 mM protease inhibitor cocktail), followed by centrifugation for 10 min at top speed. 20 µg of protein extract was mixed with 6 µL of 4x NativePAGE sample buffer and 0.06 µL of NativePAGE 5% G-250 Sample Additive (Invitrogen). 32 µL of total sample was loaded and run on a NativePAGE 3%-12% Bis-Tris gel for the primary dimension followed by incubation in 1% SDS for 15 min. Denatured gel strips were separated at constant polyacrylamide concentration (7.5%), as suggested by the supplier (Invitrogen). SDS-PAGE followed by immunoblot with anti-Myc or anti-HA was performed.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.03.018.

**AUTHOR CONTRIBUTIONS**

D.T.N.T., E.-H.C., R.S., J.L.D., D.W., and E.C. conceived, designed, and coordinated the research. D.T.N.T., E.-H.C., A.H.-M., M.D., and E.C. conducted the experiments. D.T.N.T., E.-H.C., and E.C. analyzed the data. D.T.N.T., J.L.D., D.W., and E.C. wrote the paper with help from all authors.

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