A truncated singleton NLR causes hybrid necrosis in Arabidopsis thaliana

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Abstract
Hybrid necrosis in plants arises from conflict between divergent alleles of immunity genes contributed by different parents, resulting in autoimmunity. We investigate a severe hybrid necrosis case in Arabidopsis thaliana, where the hybrid does not develop past the cotyledon stage and dies three weeks after sowing. Massive transcriptional changes take place in the hybrid, including the upregulation of most NLR disease resistance genes. This is due to an incompatible interaction between the singleton TIR-NLR gene DANGEROUS MIX 10 (DM10), which was recently relocated from a larger NLR cluster, and an unlinked locus, DANGEROUS MIX 11 (DM11). There are multiple DM10 allelic variants in the global A. thaliana population, several of which have premature stop codons. One of these, which has a truncated LRR-PL region, corresponds to the DM10 risk allele. The DM10 locus and the adjacent genomic region in the risk allele carriers are highly differentiated from those in the non-risk carriers in the global A. thaliana population, suggesting that this allele became geographically widespread only relatively recently. The DM11 risk allele is much rarer and found only in two accessions from southwestern Spain – a region from which the DM10 risk haplotype is absent – indicating that the ranges of DM10 and DM11 risk alleles may be non-overlapping.

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Introduction

Hybrid necrosis, a common form of hybrid incompatibility in plants, is caused by conflicting elements of the plant immune system originating from different parental accessions. These pairwise deleterious epistatic interactions usually involve at least one nucleotide binding site-leucine-rich repeat (NLR) protein (Bomblies et al. 2007; Alcázar et al. 2009; Yamamoto et al. 2010; Chae et al. 2014; Sicard et al. 2015; Deng et al. 2019). NLRs function as intracellular immune receptors, similarly to NOD/CARD proteins in animals, and play a major role in plant innate immunity (Maekawa, Kufer, and Schulze-Lefert 2011; Jones, Vance, and Dangl 2016). The constant co-evolutionary arms-race between plants and their pathogens has led to a high diversification of many elements of the plant immune system, including NLRs (Jones and Dangl 2006; Dodds and Rathjen 2010). Hybrid necrosis can be viewed as collateral damage resulting from, sometimes relatively minor, sequence differences between NLR alleles. This phenomenon can limit the possible NLR allele combinations found in an individual plant (Chae et al. 2014).

Plant NLRs are multidomain proteins usually composed of N-terminal Toll/Interleukin-1 receptor/Resistance protein (TIR), coiled-coil (CC) or RESISTANCE TO POWDERY MILDEW 8 (RPW8) domains, a central nucleotide-binding site (NBS) and C-terminal leucine-rich repeats (LRRs) (Meyers et al. 2003; Shao et al. 2016). The N-terminal domain is usually thought to be involved in signal transduction, while the NBS domain can act as a molecular ON/OFF switch (Bentham et al. 2017). The LRR domain is highly variable and consists of multiple repeats of 20-30 amino acid stretches that are often responsible for direct or indirect pathogen effector recognition as well as NLR auto-inhibition (Ade et al. 2007; Krasileva, Dahlbeck, and Staskawicz 2010; Steinbrenner, Goritschnig, and Staskawicz 2015). In addition, many TIR-NLRs carry a post-LRR (PL) domain, which is involved in pathogen effector recognition (Van Ghelder and Esmenjaud 2016; Martin et al. 2020).

Approximately half of all NLR genes in a given A. thaliana accession are found in multi-gene clusters, which are unevenly distributed across the genome (Meyers et al. 2003; Van de Weyer et al. 2019). Tandem duplication events are common in NLR clusters, and duplicate genes are a major source of genetic variation, since they often experience relaxed selection and enable neofunctionalization (Ohno 1970; Force et al. 1999; Lynch and Conery 2000; Conant and Wolfe 2008). Sequence homogenization through intergenic exchange among cluster members is greatly reduced when an NLR gene is translocated away from its original cluster to an unlinked genomic region, thereby preserving its original function or potentially developing a new one (Baumgarten et al. 2003; Leister 2004). For NLRs, neofunctionalization of duplicated or translocated genes can expand the repertoire of pathogen effectors an individual plant is able to recognize (Botella et al. 1998; Michelmore and Meyers 1998; Holub 2001; Kim et al. 2017).
Genome-wide analysis of structural variation across eight high-quality A. thaliana genomes identified rearrangement hot spots coinciding with numerous multi-gene NLR clusters (Jiao and Schneeberger 2020), including the previously described DANGEROUS MIX (DM) loci, which are causal for hybrid necrosis (Bomblies et al. 2007; Chae et al. 2014). This raises the possibility that accelerated evolution associated with genomic rearrangements contribute to the generation of incompatibility alleles, pointing to genomic architecture as a driver of hybrid incompatibility. So far, over a dozen NLR loci with hybrid necrosis alleles are known from multiple plant species. Curiously, even though singletons account for about a quarter of NLRs in different species (Jacob, Vernaldi, and Maekawa 2013), none of the causal NLR loci identified so far is a singleton NLR, here defined as a physical single-gene NLR and not to be confused with a functional singleton (Adachi, Derevnina, and Kamoun 2019). Most, but not all, well-characterized singleton NLRs, such as RPM1 and RPS2 in A. thaliana, show ancient balanced polymorphisms that maintain active and inactive alleles at intermediate frequencies in natural metapopulations (Stahl et al. 1999; Caicedo, Schaal, and Kunkel 1999; Mauricio et al. 2003; Allen et al. 2004; MacQueen, Sun, and Bergelson 2016). Thus, with less functional diversity, and beneficial alleles often being relatively common, one would indeed expect that singleton NLRs are underrepresented among hybrid necrosis loci.

Here, we are investigating a case of severe hybrid necrosis, where hybrid plants do not develop past the cotyledon stage, become necrotic, and die three weeks after sowing. Extensive transcriptional changes occur in the hybrid, including the induction of most NLR genes. Through a combination of QTL analysis and GWAS, we identified two new incompatibility loci, DANGEROUS MIX 10 (DM10), a TIR-NLR on chromosome 5, and DM11, an unlinked locus on chromosome 1, as causal for incompatibility. DM10 is an unusual hybrid incompatibility locus because it is a singleton NLR that arose after A. thaliana speciation through interchromosomal transposition from the RLM1 cluster, which confers resistance to Leptosphaeria maculans, that causes blackleg disease in Brassica species (Staal et al. 2006; Guo et al. 2011). The causal allele has a premature stop codon that removes the C-terminal quarter of the protein, which includes part of the LRR-PL region, indicating that substantial NLR truncations can lead to hybrid incompatibility.

Results

A particularly severe case of hybrid necrosis: Cdm-0 x TueScha-9

Eighty A. thaliana accessions have previously been intercrossed with the goal of identifying hybrid incompatibility hot spots (Chae et al. 2014). A particularly severe case was observed in the crosses between Cdm-0 and five other accessions: TueScha-9, Yeg-1, Bak-2, ICE21 and Leo-1. The F1 progeny of these two parents did not develop past the cotyledon stage, even at temperatures that suppress hybrid necrosis in most other cases.
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(Chae et al. 2014), and severe necrosis developed during the second week after sowing, followed by complete withering in the third week (Fig 1A).

To obtain insights into the transcriptional changes in the hybrid, we performed RNA-seq on the parental accessions Cdm-0 and TueScha-9, as well as in F1 hybrid plants 10 days after germination, when the hybrid was already slightly stunted, but before there were visible signs of necrosis (Fig 1B). We observed massive transcriptional changes, in which around half of all 20,000 detectable genes (Fig S1A) were differentially expressed in the hybrid when compared to either parent (Fig 1C, S1B, Table S1). This represents one third of the entire A. thaliana transcriptome (Klepikova et al. 2016). A principal component analysis (PCA) showed that most of the variance in gene expression is driven by the difference between the parents and the hybrid (PC1: 83 %) (Fig 1D). In addition, we generated in silico hybrids (see Methods) and compared these with the biological F1 hybrids through a PCA. This confirmed that gene expression in the F1 hybrid is not an additive result of expression in the two parental accessions (Fig S1C). Next, we carried out a Gene Ontology (GO) analysis using the top 1,000 differentially expressed genes (DEGs) from a comparison of the F1 hybrids and the mid-parental values (MPV) (Table S2). “Defense response” and “salicylic acid biosynthesis” were the categories with the highest number of DEGs in the hybrid versus MPV comparison (Fig 1E, Table S2).

Since the F1 hybrid displayed signs of an increased pathogen defense response, we analyzed the expression of a set of marker genes for defense-associated phytohormones such as jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) (Papadopoulou et al. 2018), as well as early pathogen response genes induced by both cell surface receptors and NLRs (Ding et al. 2020) (Table S3). Genes involved in SA biosynthesis and signaling, such as EDS1, ICS1, EDSS, PAD4, PBS3, CBP60 and FMO1, were strongly overexpressed in F1 hybrid plants, in concordance with the GO analysis, as was the SA-induced camalexin biosynthesis gene CYP71A13. The expression of genes encoding transcription factors WRKY46 and WRKY51 and of the late immune response gene PR1 was also increased in the hybrid (Fig 1F, Table S3). In contrast, the expression of genes required for JA-mediated resistance, such as MYC2 or DDE2, or genes involved in ET signaling, such as ETR1, ERF1 and EIN3, changed to a lesser extent in the F1 hybrid, similar to control genes ACT7 and TUB2 (Fig 1F, Table S3).

Since an increase in NLR expression has been linked to autoimmunity (Stokes, Kunkel, and Richards 2002; Mackey et al. 2003; Palma et al. 2010; Lai and Eulgem 2018), and since some NLRs are upregulated when SA levels rise (Shirano et al. 2002; Yang and Hua 2004; Tan et al. 2007; Mohr et al. 2010; MacQueen and Bergelson 2016), we set out to investigate NLR expression levels in the hybrid. Out of a set of 166 NLRs found in the Col-0 genome, 150 were expressed in at least one of the three genotypes studied, and 128 were significantly ([log2FoldChange] >1, padj value < 0.01) differentially expressed in at least one genotype comparison (Fig S2D, Table S4). From these 128 NLRs, all but one were differentially expressed when comparing the hybrid
with either parental accession (Fig S1D). NLRs were mostly upregulated in the F₁ hybrid: of the 95 NLRs with significant expression changes in the hybrid versus the MPV, all but three were overexpressed (Fig 1G, S1E, Table S1). When the F₁ hybrid was compared to the parents, the expression of individual NLRs largely followed the same pattern (Fig S1E-G), this was not the case when comparing the two parents (Fig S1H). The fraction of genes overexpressed in the F₁ hybrid was similar for the different NLR classes as well as between singleton and clustered NLRs (Fig 1G, S1F-H, Table S3).

**Fig 1. RNA-seq analysis of Cdm-0 x TueScha-9 hybrids.**

A. At 21 days, the Cdm-0 x TueScha-9 F₁ hybrid is necrotic. Plants were grown at 16°C. Scale bar represents 1 cm. B. Examples of a 10-day old Cdm-0 x TueScha-9 F₁ hybrid and parental accessions harvested for RNA-seq. Plants were grown at 23°C. Scale bar represents 1 cm. C. Intersection of DEGs between the F₁ hybrid and parents. D. PCA of gene expression values. The main variance is between the F₁ hybrid and parents. Each dot indicates one biological replicate, with six per genotype. E. REVIGO Gene Ontology treemap. Size of the square represents -log₁₀(p value) of each GO term. F. log₁₀(normalized read count) of defense-related marker genes of the hybrid and the parents. G. Differences in expression between the F₁ hybrids and the mid-parental values (MPV) of NLR genes, with 128 significantly (|log₂FoldChange| >1, padj value < 0.01) differentially expressed in at least one genotype comparison.
QTL mapping of DM10 and DM11 in a triple-hybrid cross

Having found that a very large fraction of NLR genes is upregulated in the Cdm-0 x TueScha-9 hybrid, we wondered whether hybrid necrosis in this case was due to global NLR regulators (Li, Pennington, and Hua 2009; Zhai et al. 2011; Shivaprasad et al. 2012; Gloggnitzer et al. 2014; Sicard et al. 2015), or to NLRs, as in other hybrid necrosis cases. We therefore proceeded to map the underlying causal loci via quantitative trait locus (QTL) analysis. Since the F1 hybrid seedlings died very young, we could not directly generate a segregating F2 mapping population (Bomblies et al. 2007; Chae et al. 2014; Barragan et al. 2019). Instead, we designed a triple-hybrid cross (Cooper et al. 2019) and first generated two sets of heterozygous plants by crossing Cdm-0 and TueScha-9 separately to a third, innocuous background, the Col-0 reference accession. We then intercrossed these Cdm-0/Col-0 and TueScha-9/Col-0 plants (Fig 2A). In the resulting pseudo-F2 generation, we collected both normal and necrotic plants and individually genotyped them by RAD-seq (Rowan et al. 2017). For QTL mapping, we focused on polymorphic markers between Cdm-0 and TueScha-9, including markers overlapping with the Col-0 reference (Fig 2B), and also analyzed polymorphic markers for each accession independently (Fig S2A, B). We identified two genomic regions that interact epistatically to cause the severe hybrid necrosis phenotype. We called the QTL on chromosome 5 (23.35 to 24.45 Mb) DM10, and the QTL on chromosome 1 (21.55 to 22.18 Mb) DM11. Both intervals contain NLRs but no clear candidates for global NLR regulators, so we chose to focus on NLR genes. In the DM10 mapping interval, one NLR is present, At5g58120, while the DM11 interval was NLR-rich and encompassed 10 NLRs in Col-0 (Table S5). Loci in the interval include the highly polymorphic RPP7 cluster of CC-NLR genes (McDowell et al. 2000; Guo et al. 2011; Li et al. 2020), as well as two CC-NLR singleton genes, CW9 (At1g59620) and At1g59780 (Meyers et al. 2003). To identify potential differences between Col-0 and Cdm-0 in the DM11 interval, we generated a PacBio long-read-based genome assembly of this accession (Table S6). Notably, most chromosome arms were assembled in single contigs, including the long arm of chromosome 1, where the DM11 mapping interval is located (Fig S3). Since at the time the full Cdm-0 annotation was not yet available, we manually annotated homologs of NLR genes corresponding to the genomic region that spans from At1g56510 to At1g64070 in Col-0, which includes the DM11 mapping interval as well as neighboring NLRs. Like Col-0, Cdm-0 carries groups of both clustered and singleton NLRs, adding up to a total of 21 NLRs, compared to 28 NLRs in Col-0 (Fig 2C, Table S5).

To pinpoint DM11 candidate genes, we sought to identify additional accessions that had similar alleles as Cdm-0 at DM11 candidate loci by creating Neighbor-Joining (NJ) trees (Fig S2C) and PCA plots (Fig S2D, Table S7), using sequences from the 1001 Genomes Project (1001 Genomes Consortium 2016). IP-Cum-1 was the accession most similar to Cdm-0 for the whole DM11 mapping interval, and when we crossed it to TueScha-9, Cdm-0 x TueScha-9-like hybrid necrosis was observed (Fig 2D). Eleven other accessions that were less
closely related to Cdm-0 in this genomic interval did not produce necrotic F₁ hybrids (Table S7). Because accessions Isitsu-1 and ICE134, like Cdm-0, lack a transposable element that is present in most RPP7 (At1g58602) alleles (Tsuchiya and Eulgem 2013), we also crossed these two accessions to TueScha-9, but no hybrid necrosis was observed (Table S7). Artificial miRNAs (amiRNAs) (Schwab et al. 2006) targeting different members of the RPP7 cluster were previously designed to perform rescue experiments for other cases of hybrid necrosis (Chae et al. 2014; Barragan et al. 2019); although predicted to target all members of the Cdm-0 RPP7 cluster, neither these nor amiRNAs targeting CW9Cdm-0 or At1g59780Cdm-0 suppressed hybrid necrosis (Table S8). Lastly, a genomic CW9Cdm-0 fragment was unable to induce hybrid necrosis when introduced into TueScha-9 (Table S18).

Fig 2. QTL mapping with a triple-hybrid cross. A. Creation of a Cdm-0 x TueScha-9 mapping population. B. QTL analysis from polymorphic Cdm-0 and TueScha-9 markers. QTL peaks are found on chromosome 5 (23.35-24.45 Mb), DM10, and chromosome 1 (21.55-22.18 Mb), DM11. The horizontal lines indicate 0.05 significance threshold established with 1,000 permutations. C. Comparison and distribution of candidate DM11 NLR genes between At1g56510 and At1g64070 on chromosome 1. Gene IDs in grey are present in Col-0 but not in Cdm-0, gene duplications are marked in green and inversion events in orange. Asterisks indicate significant (|log₂FoldChange|>1, padj value < 0.01) gene expression changes in the F₁ hybrid when compared to the MPV. D. Cdm-0 x TueScha-9 and IP-Cum-1 x TueScha-9 hybrids. Plants are two weeks old and were grown at 16°C. Scale bar represents 1 cm.

Being aware that the precision of QTL mapping in NLR-rich regions can be affected by structural variation, we also tested NLRs adjacent to the DM11 mapping interval. The RLM1 cluster is highly similar among Cdm-0 and IP-Cum-1, both of which carry the causal DM11 allele in addition, some cluster members show an increased expression in the F₁ hybrid, which is sometimes the case for causal NLRs (Bomblies et al. 2007) (Fig S2E). We therefore tested six of the seven RLM1 cluster members via Nicotiana benthamiana co-expression with DM10TueScha-9 (see Fig 4 for cloning of causal DM10 allele), but none induced a hypersensitive response (HR)
Six accessions with a similar RLM1 locus to that of Cdm-0 and IP-Cum-I were crossed with TueScha-9, but no necrosis was observed (Table S7). Finally, because At1g57650 was strongly upregulated among DM11 NLR candidate genes, we tested it with DM10TueScha-9 in N. benthamiana, but again no HR was observed (Fig S2E, Table S4, S5). This may indicate that DM11 is either an NLR that was not tested, or not an NLR at all. Other DM11 candidates may include any of the genes in this interval that encode proteins that are not annotated as NLRs but have a TIR or LRR domain (Tables S9-S11). Note that some Col-0 NLRs that had no homologs in the interval from At1g56510 to At1g64070 in Cdm-0 attracted non-specific RNA-seq reads, most likely because there are homologs elsewhere in the Cdm-0 genome (Table S5).
acessions. The significance threshold (Bonferroni correction, 5% familywise error) is indicated as a horizontal dotted line (same in C). C. Close-up of the region highly associated with hybrid necrosis; SNPs with a 1:1 association marked in teal. Asterisks indicate such 1:1 associations in At5g58120, ROS3 and PHOT2; see also E and F. SNP positions are given in Table S13. D. Linkage disequilibrium (LD) across a 50 kb region in chromosome 5. Strong linkage is observed from At5g58090 to At5g58140. E. LD across the same 50 kb region as in D, with a subset of markers from 80 accessions crossed to Cdm-0. Asterisks indicate markers highlighted in C. F. LD across a 50 kb region with the same markers as in E, but for the five risk accessions only. Higher LD is seen here than in E.

Fine-mapping of DM10 using genome-wide association studies

In the original collection of 6,409 crosses among 80 accessions (Chae et al. 2014), four accessions in addition to TueScha-9 produced severe hybrid necrosis when crossed to Cdm-0: Yeg-1, Bak-2, ICE21 and Leo-1. Together with TueScha-9, these represent much of the Eurasian range of the species, both geographically and genetically; six of the nine previously identified admixture groups (1001 Genomes Consortium 2016) are present in these five risk accessions (Fig 3A, Table S12). Given the diversity of the five incompatible accessions, and knowing that most, but not all, causal genes for hybrid incompatibility are NLRs, we attempted to narrow down causal DM10 candidate genes by GWAS, with Cdm-0-dependent F1 necrosis as a binary trait (Grimm et al. 2016). We discovered a remarkably high association between this phenotype and several closely linked markers on the bottom of chromosome 5, with corrected p values as low as 10^{-38}. In addition, 79 SNPs showed a one-to-one association with the necrotic phenotype, resulting in -log_{10} p values of 0 (Fig 3B, Table S13). The markers with the strongest associations tagged three loci: At5g58120, encoding a TIR-NLR without known function, ROS3 (At5g58130), encoding an enzyme involved in DNA demethylation (Zheng et al. 2008), and PHOT2 (At5g58140), encoding a blue light receptor that mediates phototropism (Harada, Sakai, and Okada 2003) (Fig 3C, Table S13). These three loci are genetically similar among the five risk accessions, yet differentiated from the other 75 accesses used for GWAS (Fig S4A-C). Looking at linkage among loci in this genomic region, we could see that, when taking all 80 accessions into account, six loci (At5g58090-Atg58140) belong to one large linkage block, in which ROS3 and PHOT2 are under tight linkage and the TIR-NLR At5g58120 constitutes a separate linkage block (Fig 3D). Notably, in the five accessions causing hybrid incompatibility, stronger linkage is observed in this region than that seen among the same markers from all 80 accessions (Fig 3E, F). In the risk accessions, At5g58120, ROS3 and the proximal part of PHOT2 form one linkage block, while SNPs located in the distal half of PHOT2 are found in a separate linkage block, rendering At5g58120 and ROS3 as primary candidates for causality in hybrid necrosis (Fig 3F).

DM10, a singleton TIR-NLR, as cause of severe hybrid necrosis

Having candidate genes for DM10, we next sought to experimentally test their causality for severe hybrid necrosis. Genomic fragments of the TIR-NLR At5g58120 and ROS3, from both Col-0 and TueScha-9, were
introduced into Cdm-0 plants. A 4.8 kb genomic fragment containing At5g58120\textsuperscript{TueScha-9} recapitulated the Cdm-0 x TueScha-9 hybrid necrosis phenotype (Fig 4A, Table S14). At5g58120 is henceforth called DM10. When DM10\textsuperscript{TueScha-9} was introduced into a Col-0 background and the resulting T\textsubscript{1} plants were subsequently crossed to Cdm-0, we also observed the hybrid incompatibility phenotype in the F\textsubscript{1} progeny (Fig 4B). DM10\textsuperscript{Col-0}, ROS3\textsuperscript{TueScha-9} and ROS3\textsuperscript{Col-0} did not produce any necrosis when introduced into a Cdm-0 background (Table S14). We also observed that, when infiltrated in N. benthamiana leaves and overexpressed under an EtOH-inducible promoter, both DM10\textsuperscript{Col-0} and DM10\textsuperscript{TueScha-9} were able to trigger cell death, which was not the case when DM10\textsuperscript{Col-0} and DM10\textsuperscript{TueScha-9} were expressed under the control of their native promoters (Fig 4C). The cell death-triggering activities under forced overexpression in the heterologous N. benthamiana system indicate that these NLRs are competent in immune signaling and, in the case of DM10\textsuperscript{TueScha-9}, this is not abolished by its substantial truncation.

**Fig 4. Experimental identification of DM10.** A. Recapitulation of hybrid necrosis in 25-day old Cdm-0 T\textsubscript{1} plants transformed with the indicated genomic fragment from TueScha-9 or Col-0. Representative phenotype and total number of T\textsubscript{1} plants examined given on top. Plants were grown at 16°C. Scale bar represents 1 cm. B. The same DM10\textsuperscript{TueScha-9} genomic fragment as in A was introduced into Col-0, and T\textsubscript{1} plants were crossed to Cdm-0. The F\textsubscript{1} hybrid phenotype was recapitulated (magenta arrows). Plants were 18 days old and grown at 16°C. Scale bar represents 1 cm. C. Infiltration of N. benthamiana leaves with the indicated constructs. Overexpression of either DM10\textsuperscript{TueScha-9} or DM10\textsuperscript{Col-0} under an EtOH inducible promoter (pEtOH-ind) triggered cell death, while expression from their native promoter (pDM10) did not. Images were taken 7 days after infection.

**Prevalence and genetic differentiation of the DM10 risk allele in the global A. thaliana population**

To study natural variation across different DM10 alleles, 73 alleles belonging to accessions originating from across A. thaliana’s native range were extracted from preliminary short- and long-read genome assemblies available in-house (Fig S5A, Table S15). A Maximum-Likelihood (ML) tree of these alleles showed that there are multiple well-supported DM10 clades (Fig S5B), and that variation between DM10 proteins was
most prevalent at the C-terminal end (Fig 5A, Fig S5C, Table S16). Ten alleles were predicted to produce proteins truncated at three different points. Four accessions, including TueScha-9, the original DM10 risk allele carrier, share the same stop codon (Fig 5B, S5B), removing three LRRs and the post-LRR domain (PL). Three short motifs have been previously recognized as being conserved in PLs of different NLRs (Van Ghelder and Esmenjaud 2016) (Fig 5B, Fig S5C). The DM10 PL has a variant of the first of these motifs, in a degenerate form. Five accessions had shorter, 335 amino acid long DM10 proteins; in these, the NBS domain was truncated, lacking motifs which are important regulators of NLR activity (Bendahmane et al. 2002; Sueldo et al. 2015; Bentham et al. 2017) (Fig 5B, Fig S5C). These five accessions carrying short DM10 alleles included Cdm-0 and IP-Cum-1, which also carry DM11 risk alleles. This implies that the short Cdm-0-like DM10 variants do not interact with DM11 to produce hybrid necrosis. The shortest predicted DM10 protein, found in the Sha accession, is only 90 amino acids long and is truncated midway through the TIR-2 motif (Fig 5B, Fig S5C). There are full-length DM10 alleles without any non-synonymous differences to DM10<sup>Cdm-0</sup> and DM10<sup>Sha</sup>, and that are distinguished from these only by truncation. Similarly, the full-length DM10<sup>Col-0</sup> and the truncated DM10<sup>TueScha-9</sup> proteins differ only at 3% of shared sites, which is low for within-species variation among NLR alleles (Van de Weyer et al. 2019). Furthermore, not only the coding sequence, but also the sequence after the premature stop codon of the short DM10 alleles, is highly similar to that of full-length alleles. This lack of signs of pseudogenization suggests that the truncations occurred relatively recently. As is typical for NLRs (Mondragón-Palomino et al. 2002; Ruggieri, Nunziata, and Barone 2014), Ka/Ks values above 1 were found in the LRR domain when comparing DM10<sup>Col-0</sup> and DM10<sup>TueScha-9</sup> (Fig 5C). In contrast, DM10<sup>Col-0</sup> and DM10<sup>Lerik1-3</sup>, which are both full-length DM10 proteins but from different clades, are more differentiated in TIR and NB-ARC domains, although Ka/Ks values above 1 are also restricted to the LRR domain (Fig S5D).

Next, to assess how common the DM10 risk allele is in the global <i>A. thaliana</i> population, we again turned to the 1001 Genomes collection (1001 Genomes Consortium 2016). Since DM10, ROS3 and the proximal part of PHOT2 were strongly linked in accessions carrying the DM10 risk allele, we focused on this region, which contained 785 SNPs. In a NJ tree, all five confirmed DM10 risk allele carriers were found in the same branch, which included 95 other accessions (Fig 5D, Table S17). In a PCA of this region, these 100 accessions were clearly separated from the rest (Fig 5E), which was not the case in a whole-genome PCA (Fig S5E), indicating that population structure is not the main driving force separating risk from non-risk allele carriers. To experimentally confirm that sequence was predictive of interaction with the DM11 risk allele, 25 of the 100 accessions were crossed to Cdm-0 (Fig S5B, Table S17). All but IP-Alm-0 produced hybrid necrosis. Notably, while DM10 from IP-Alm-0 is 99.2% identical with DM10<sup>TueScha-9</sup>, it does not have the LRR truncation (Fig S5B). This implies that the truncation in DM10 risk alleles is likely responsible for incompatibility, and not individual amino acid changes. Ten random accessions not predicted to carry the DM10 risk allele were
crossed to Cdm-0 as a control; as expected, none produced hybrid necrosis (Table S17). Similarly, we investigated how common the other two DM10 truncations are in the global A. thaliana population. The shortest Sha-like DM10 allele was found in 29 accessions, while the Cdm-0-like truncation is more common, although not as common as the DM10 risk allele, and was found in 67 accessions (Table S17).

Fig 5. DM10 natural variation. A. Amino acid alignment of 73 DM10 proteins color-coded by its conservation score (Armon, Graur, and Ben-Tal 2001). B. Comparison between different DM10 proteins (aligned with A). C. Ka/Ks ratios between DM10Col-0 and DM10Toscha-9. D. NJ tree of a region including DM10, ROS3 and PHOT2 sequences from the 1001 Genomes Project (1001 Genomes Consortium 2016). Branch lengths in nucleotide substitutions are indicated. Accessions carrying the DM10 risk alleles group together in a branch (magenta), risk accessions crossed to Cdm-0 are highlighted. E. PCA. Accessions carrying the predicted DM10 risk (magenta) versus non-risk (grey) alleles are clearly separated in PC2. Risk accessions crossed to Cdm-0 are outlined in black. F. π, Tajima’s D and FST for DM10 (magenta), the DM10 linkage block comprising At5g58090-At5g58140 (green) and surrounding genomic regions.

In a 200 kb region around DM10, nucleotide diversity (π) was highest, up to 0.015, in the distal half of DM10, encoding the more polymorphic LRR domain (Fig 5F). However, in comparison with other TIR-NLRs present
in most or all accessions, overall DM10 nucleotide diversity was not uncommon (Van de Weyer et al. 2019). Values for Tajima’s D reached 4.8 in the proximal half of DM10, hinting at multiple DM10 alleles being prevalent at intermediate frequencies in the global A. thaliana population, as is often the case for NLRs (Stahl et al. 1999; Caicedo, Schaal, and Kunkel 1999; Bakker et al. 2006; Karasov et al. 2014). Lastly, the fixation index (\(F_{ST}\)) between 98 accessions with predicted DM10 risk alleles (excluding IP-Alm-0 and RAD-21, which did not have truncated LRR domains) and 1,037 non-risk allele carrying accessions, peaked at 0.88 across the DM10 linkage block (Fig 3D-F, Fig 5F). This was the only peak detected both across the entire chromosome 5 (Fig SSF) and the whole genome. Inside this block, a drop in \(F_{ST}\) is seen over the proximal half of DM10, which is consistent with this region being similar between risk and some non-risk alleles (Fig 5C).

Taken together, these results show that there are multiple DM10 alleles in the global A. thaliana population, three of which are predicted to result in truncated proteins due to the presence of premature stop codons, one of which is the DM10 risk allele. Notably, the DM10 risk allele is not only relatively common and genetically differentiated in our GWAS population, but also in the global A. thaliana population.

**No documented co-occurrence of DM10 and DM11 risk alleles in the global A. thaliana population**

Looking at the geographical distribution of accessions carrying different DM10 alleles with premature stop codons, we observed that both the Cdm-0-like DM10 allele as well as the risk DM10 allele were found at similar densities throughout A. thaliana’s native range, while the Sha-like DM10 allele was mainly found towards the eastern part of the species’ distribution (Fig 6A, Table S17). In the case of the DM10 risk allele, the one exception to where this allele was found, was the southwestern part of Spain and Portugal, even though A. thaliana has been heavily sampled in this region (1001 Genomes Consortium 2016). The fact that the only two DM11 risk carriers identified so far, Cdm-0 and IP-Cum-1, are found in southwestern Spain may indicate that the DM10 and DM11 risk alleles do not geographically co-occur (Fig 6B).

To provide additional support for this assertion, we first attempted to identify more DM11 risk carriers in Spain and Portugal. We crossed TueScha-9, a DM10 risk allele carrier, to 24 accessions from these two countries, which were found at different geographical distances from the two DM11 risk carriers Cdm-0 and IP-Cum-1, as well as from accessions carrying the DM10 risk allele (Table S7). No hybrid necrosis was observed in any of the resulting F1 progeny (Fig 6B, Table S7). This, together with our aforementioned attempts to find additional DM11 carriers among accessions that are closely related in the DM11 genomic region to Cdm-0 and IP-Cum-1, indicates that the DM11 risk allele is rare and potentially only found in southwestern Spain, a region where the DM10 risk allele appears to be absent.
Fig 6. Geographical distribution of DM10 and DM11 alleles. A. Geographical distribution of 1001 Genomes Project accessions (1001 Genomes Consortium 2016) carrying the DM10 risk (magenta), non-risk (grey) alleles, Sha-like non-risk (orange) and Cdm-0-like non-risk (green) alleles. Rectangle zooms into the region shown in B. B. Distribution of 1001 Genomes Project accessions (grey) in Spain and Portugal, carrying the DM10 (magenta) and DM11 (red) risk alleles, as well as accessions carrying DM11 non-risk alleles (brown) which were crossed to TueScha-9.

Origin of the DM10 NLR singleton locus through a recent interchromosomal relocation event out of the RLM1 cluster

In the A. thaliana Col-0 reference genome, we identified nine NLR genes closely related to DM10. Seven of these make up the RLM1 cluster on chromosome 1, and two others, At2g16870, At4g14370 are dispersed singletons. In the related species Arabidopsis lyrata and Brassica rapa, we identified a further 20 DM10/RLM1 homologs (Fig 7A). As in A. thaliana, the cluster homologous to the A. thaliana RLM1 cluster in these two species (not to be confused with the RLM1 locus that provides resistance to blackleg disease in Brassica (Delourme et al. 2008; Fu et al. 2019)) underwent within-species duplication and inversion events (Fig 7B). Most RLM1 members from A. thaliana have a clear one-to-one homolog in A. lyrata, so the expansion of the RLM1 cluster must have occurred before the two species diverged (Beilstein et al. 2010). The A. lyrata homologs of At2g16870 and At4g14370, 480565 and 493465, are found in a different chromosome than the main RLM1 cluster (Fig 7C). This is not the case for the DM10 homolog from A. lyrata, 875509, which is located inside the main RLM1 cluster (Fig 7C). This indicates that DM10 was relocated away from the main RLM1 cluster to another chromosome and that this occurred after A. lyrata and A. thaliana diverged.
Fig 7. RLM1 locus in B. rapa, A. lyrata and A. thaliana reference genomes. A. ML tree of the NBS domain (CDS) of RLM1 cluster members from B. rapa, A. lyrata and A. thaliana, with the NBS domain of C. elegans CED-4 as outgroup (C35D10.9a.1). 1,000 bootstrap replicates were performed, values are shown on each branch. Branch lengths in nucleotide substitutions are indicated. The same color was chosen for genes in neighboring branches with bootstrapping values above 70. Diagonal lines indicate a gap in the tree branches. B. RLM1 cluster members and their homologs in B. rapa, A. lyrata and A. thaliana. Color-coding the same as in A, genes in grey are truncated, arrows represent size of NLR loci. Diagonal lines indicate a positional gap along the chromosome, the length of the gap is indicated. C. Heatmaps of NLR densities across the three genomes. Window sizes were calculated by dividing the length of the longest chromosome by 100. RLM1 cluster and
closely related singletons indicated. DM10 in *A. thaliana* and its homolog in *A. lyrata*, 875509 are highlighted in magenta.

**Discussion**

Over ten causal genes for hybrid necrosis have been identified in *A. thaliana* and other plants (Krüger et al. 2002; Bomblies et al. 2007; Alcázar et al. 2009; Jeuken et al. 2009; Yamamoto et al. 2010; Chen et al. 2014; Chae et al. 2014; Todesco et al. 2014; Sicard et al. 2015; Deng et al. 2019; Sandstedt, Wu, and Sweigart 2020). In many instances, at least one of the two causal genes is an NLR, which is also the case for the Cdm-0 x TueScha-9 incompatibility. What makes this case particularly interesting is the extreme severity of hybrid necrosis, the transcriptional hyper-induction of NLR genes, and the causality of a truncated singleton NLR, DM10, which was recently relocated from a larger NLR cluster.

As with normal immune responses and autoimmune syndromes, the expression of hybrid necrosis is typically temperature-dependent, and hybrid necrosis in *A. thaliana* can usually be completely suppressed when grown above 23°C (Bomblies et al. 2007; Alcázar et al. 2009; Chae et al. 2014; Todesco et al. 2014; Świadek et al. 2017). In contrast, the extreme autoimmune response in Cdm-0 x TueScha-9 F1 seedlings cannot be rescued even by growing these hybrids at 28°C (Chae et al. 2014). In other necrotic hybrids, non-causal NLRs have been reported to be differentially expressed between hybrids and their parents (Bomblies et al. 2007; Atanasov et al. 2018), but the NLR induction seen in Cdm-0 x TueScha-9 is clearly the most extreme. For example, in the F1 progeny, 128 of 150 expressed NLRs are differentially expressed in at least one genotype comparison, with almost all being overexpressed. When we reanalyzed published data from another, relatively strong hybrid necrosis case, we found 104 out of 166 NLR genes to be differentially expressed, yet both extreme as well as the mean of overexpression was lower than in Cdm-0 x TueScha-9 hybrids (Fig S6 and Tables S1-S4). In addition, the specific NLR genes that are overexpressed differ in both cases, indicating that specific NLRs respond differently depending on their genetic background (Fig S6 and Table S4). Simultaneous upregulation of several NLR genes has been observed after exposure to biotic (Zipfel et al. 2004; Tan et al. 2007; Ribot et al. 2008; Mohr et al. 2010; Yu et al. 2013; Sohn et al. 2014; Chen et al. 2015; Mine et al. 2018; Steuernagel et al. 2020) and abiotic stresses (MacQueen and Bergelson 2016), but not to the extent seen in Cdm-0 x TueScha-9 hybrids. Given that elevated NLR expression levels can trigger cell death (Stokes, Kunkel, and Richards 2002; Mackey et al. 2003; Palma et al. 2010; Lai and Eulgem 2018), we expect that widespread NLR hyper-induction is a significant contributor to the strongly necrotic phenotype of Cdm-0 x TueScha-9 F1 hybrids.

NLR transcript levels are tightly controlled through a variety of regulatory mechanisms (Lai and Eulgem 2018), and large-scale upregulation of NLRs could possibly require multiple pathways. We found WRKY transcription factors to be overexpressed in the hybrids; these proteins bind to W box motifs enriched in the promoters of
multiple members of the plant immune system, including NLRs, and can induce widespread NLR expression, enhancing basal immunity (Eulgem and Somssich 2007; Pandey and Somssich 2009; Mohr et al. 2010). Two other mechanisms known to affect a broad set of NLRs are the miRNA-dependent phasiRNA production (Zhai et al. 2011; Li et al. 2012; Shivaprasad et al. 2012; Xia et al. 2015) as well as nonsense-mediated decay (NMD) (Gloggnitzer et al. 2014), both of which help to dampen NLR gene expression in the absence of pathogen threats. Repression is attenuated after an incoming pathogen is detected by the plant, enabling global NLR levels to increase (Lai and Eulgem 2018). While we have no direct evidence for transcription factors, small RNAs or NMD as contributors to aberrant NLR expression in the Cdm-0 x TueScha-9 hybrid, this exceptional hybrid necrosis case may present a good tool for comparing NLR regulation under pathogen attack with strong autoimmunity.

We found 17% of DM10 proteins encoded in a global set of A. thaliana accessions to be truncated in either their TIR, NBS or LRR domain. Similar to several full-length variants, the alleles for all three truncated proteins have intermediate frequencies and are relatively wide-spread, suggesting that they are actively maintained in the global population by balancing selection. The most common of the three truncation alleles is the DM10 risk version, which lacks three of the eight LRRs and the PL domain, and which shows evidence for its LRR domain being under diversifying selection. While the TIR domain alone can induce cell death (Swiderski, Birker, and Jones 2009; Bernoux et al. 2011), a complete NBS domain is essential in many instances (Dodds, Lawrence, and Ellis 2001; Dinesh-Kumar, Tham, and Baker 2000; Bendahmane et al. 2002; Tameling et al. 2002; Williams et al. 2011; Steinbrenner, Goritschnig, and Staskawicz 2015; Wang et al. 2015; Sueldo et al. 2015; Bernoux et al. 2016). NLRs lacking the NBS or LRR domain are not only known to retain the ability to cause cell death, but there are cases where truncated NLRs are bona fide resistance genes (Roth et al. 2017; Nishimura et al. 2017; Marchal et al. 2018). Conversely, other proteins, including at least one full-length NLR, can induce cell death through activation of naturally occurring truncated NLRs (Zhao et al. 2015; Y. Zhang et al. 2017). In the case of DM10, we do not know whether only the full-length variants or the truncated variants, or both, are functional and if they confer resistance to unknown pathogens even though their prevalence and geographical distribution suggest so. Alternatively the “less is more” hypothesis (Olson 1999) may explain the wide prevalence of truncated DM10 alleles even if these are non-functional. Minor mutations in these alleles could readily remove the premature stop codons, making them “nearly functional” alleles that could act as easily activable functional reservoirs, as previously discussed for RPM1 (Rose et al. 2012). The particular length of the risk DM10<sup>TueScha-9</sup> protein combines the autoactive tendencies associated with the partial loss of the LRR-PL domain (Qi et al. 2018) with what appear to be functional TIR and NBS domains.

Because NLR allelic diversity is often not easily captured by short-read based resequencing (Van de Weyer et al. 2019), we still do not have a good grasp on whether NLR alleles in general, and specifically beneficial alleles,
spread through the population more quickly than other adaptive alleles. The Iberian peninsula is a center of *A. thaliana* genetic diversity, with strong geographical structure across a north-south latitudinal gradient (Picó et al. 2008; Brennan et al. 2014). We observed a lack of co-occurrence between DM11 risk alleles, restricted to southwestern Spain, and DM10 risk alleles, restricted to the northern half of Spain (1001 Genomes Consortium 2016). Absence of co-occurrence between risk alleles may partly be the result of population structure: two geographical barriers potentially reducing gene flow, the Tagus river and the Central System mountains, divide populations carrying either DM10 or DM11 risk alleles. In any case, more definitive proof of the mutual exclusion of DM10 and DM11 risk alleles will require more extensive sampling of natural populations across the Iberian peninsula. Co-occurrence of hybrid incompatibility alleles in a single population has been observed before, where different alleles are maintained at intermediate frequencies, but in this case, the hybrids show a milder necrosis phenotype in the lab than Cdm-0 x TueScha-9, and no obvious phenotype in the wild (Todesco et al. 2014). The extreme necrotic phenotype caused by the DM10-DM11 interaction, which appears to be largely independent of growth conditions, makes it unlikely that the hybrid phenotype would be suppressed in the wild. In addition, since outcrossing rates of *A. thaliana* in the wild can be substantial (Bomblies et al. 2010), it is conceivable that in some areas these rates are high enough for lethal hybrids to exact a noticeable fitness cost on risk allele carriers.

An interchromosomal relocalization event of the RLM1 cluster gave rise to DM10 after *A. thaliana* speciation. Which evolutionary forces might have helped DM10 to become established on a separate chromosome, if any? NLR genes in clusters are likely to be more mutable than singletons because of illegitimate recombination (Michelmore and Meyers 1998; Baumgarten et al. 2003; Meyers, Kaushik, and Nandety 2005; Wong and Wolfe 2005; Wicker, Yahiaoui, and Keller 2007). If DM10 underwent beneficial neofunctionalization after duplication, its relocation away from the cluster might have stabilized the locus. Another possibility could be conflicts among gene cluster members. Cluster members are sometimes transcriptionally co-regulated (Yi and Richards 2007; Deng et al. 2017), so translocation away from the cluster would allow for evolution of new expression patterns for DM10. More generally, genomic relocation would enable DM10 to be subjected to different selection regimes than its cluster homologs. Either way, the fact that the genomic region surrounding DM10 – different from some other RLM1 cluster members – is a recombination cold spot (Choi et al. 2016) is consistent with our finding of high LD around the DM10 locus, especially in accessions carrying the DM10 risk allele. Together with our phylogenetic results and Tajima’s D measurements, this would seem to support the idea of stable DM10 haplotypes being particularly advantageous.

While our triple-hybrid cross enabled the identification of the DM10 and DM11 QTLs, fine-mapping was complicated by three sets of markers and two loci being involved. Genotyping around the DM11 locus to differentiate alleles from each of the three grandparents in the mapping cross was further confounded by
structural variants, which are typical for NLR-rich regions. Members of the DM10-related RLM1 cluster near the inferred DM11 QTL are in principle good hybrid necrosis candidates, because TIR domains tend to form homomeric complexes (Zhang et al. 2017; Dong et al. 2018; Martin et al. 2020); the similar TIR domains between DM10 and RLM1 members make it particularly likely that they oligomerize, which is often an important step in NLR activation. We cannot entirely exclude members of the RLM1 cluster, because we tested most of them only by co-expression with DM10 in the heterologous N. benthamiana system, and not by genetic inactivation or recapitulation in A. thaliana.

In conclusion, we have presented a severe case of hybrid necrosis in A. thaliana, where the hybrids show global NLR hyper-induction triggered by the interaction of DM10, a relocated singleton NLR gene, and DM11, an unlinked locus in chromosome 1. Comparative structure-function analysis of the truncated DM10<sup>TueScha</sup>-9 hybrid necrosis risk allele and the closely related full-length DM10<sup>Col-0</sup> allele, which does not cause hybrid necrosis, should reveal the exact contributions of LRR and PL subdomains to NLR activity. In addition, the DM10/DM11 case provides a good tool to investigate the consequences of simultaneous activation of a large fraction of NLRs. In the future, by identifying the role of different DM10 and RLM1 alleles in response to natural pathogens, one could test whether chromosomal relocation affects how evolution is acting on this group of highly related NLR genes.

**Materials and Methods**

Constructs are listed in Table S18 and primers in Table S19.

**Plant material**

Stock numbers of accessions used are listed in Supplementary Material. All plants were stratified in the dark at 4°C for 4-6 days prior to planting on soil. Late flowering accessions were vernalized six weeks under short day conditions (8 h light) at 4°C. All plants were grown in long days (16 h of light) at 16°C or 23°C at 65% relative humidity under 110 to 140 μmol m<sup>-2</sup> s<sup>-1</sup> light provided by Philips GreenPower TLED modules (Philips Lighting GmbH, Hamburg, Germany).

**RNA sequencing**

Six biological replicates of 10 day-old shoots of Cdm-0 x TueScha-9 hybrids and their parental accessions were collected. RNA was extracted as described in (Yaffe et al. 2012). The NEBNext magnetic isolation module (New England Biolabs), was used for mRNA enrichment. Sequencing libraries were prepared using NEBNext Ultra II directional RNA library kit and paired-end sequenced (150bp) in an Illumina HiSeq3000 (Illumina Inc.,
San Diego, USA) instrument. Reads were mapped against the A. thaliana reference TAIR10 using bowtie2 (v2.2.6) (Langmead and Salzberg 2012). Default parameters were chosen unless mentioned otherwise. Transcript abundance was calculated with RSEM (v1.2.31) (Li and Dewey 2011). In silico hybrids were generated to enable mid-parent value calculations: parental read files were normalized according to sequencing depth and were subsampled by randomly drawing 50% of the reads with seqtk (v2.0-r82-dirty; https://github.com/lh3/seqtk). Differential gene expression analyses were performed using DESeq2 (v1.18.1) (Love, Huber, and Anders 2014). Genes with less than ten counts over all 18 samples were removed from downstream analyses. Significant changes in gene expression between two genotypes were determined by filtering for genes with a $|\log_{2}\text{FoldChange}| > 1$ and padj value < 0.01. One read was added to all normalized read counts in Fig 1G, S2E and S6E to avoid plotting -INF values in non-expressed genes ($\log_{10}(0+1)=0$). Non-additive gene expression between Cdm-0 x TueScha-9 F1 hybrids in silico hybrids was analyzed by computing principal components based on the normalized read counts of the top 500 most variable genes across all 18 samples. Plots were generated using the R package ggplot2 (v3.2.0) (Wickham 2009) and heatmaps were plotted using pheatmap (v1.0.8) (Kolde 2012). Gene Ontology (GO) analyses were performed using AgriGO (Tian et al. 2017) using the SEA method. The GO results were visualized with REVIGO treemap (Supek et al. 2011), for clearer visualization only the top 13 and GO categories with the lowest p values were plotted in Fig 1G, the complete list of GO terms is found in Table S2.

**Genotyping-by-sequencing and QTL mapping**

$F_1$ progeny from bi-directional crosses of $F_1$ (TueScha-9/Col-0) x (Cdm-0/Col-0) was used as a mapping population. The seedlings showing the hybrid necrosis phenotype vs. those that did not, were genotyped individually in a 1:1 ratio. Plants were 10 days old when collected. Genomic DNA was extracted with CTAB (cetyl trimethyl ammonium bromide) buffer (Doyle and Doyle 1987) and then purified through chloroform extraction and isopropanol precipitation (Ashktorab and Cohen 1992). Genotyping-by-Sequencing (GBS) using RAD-seq was used to genotype individuals in the mapping populations with KpnI tags (Rowan et al. 2017). Briefly, libraries were single-end sequenced on a HiSeq 3000 instrument with 150 bp reads. Reads were processed with Stacks (v1.35) (Catchen et al. 2013) and mapped to TAIR10 with bwa-mem (v0.7.15) (H. Li 2013), variant calling was performed with GATK (v3.5) (McKenna et al. 2010). QTL was performed using R/qtl (Broman et al. 2003) with the information from 348 $F_2$ individuals from 4 independent lines of this segregating population and 6179 markers.

**De novo genome assembly and annotation**
The Cdm-0 accession (ID 9943; CS76410) was grown as described above. To reduce starch accumulation, 3-week-old plants were put into darkness for 30 h before harvesting. Sixteen grams of flash frozen leaf tissue were ground in liquid nitrogen and nuclei isolation was performed according to (Workman et al. 2018) with the following modifications for A. thaliana: eight independent reactions of two grams each were carried out, and the filtered cellular homogenate was centrifuged at 7,000 x g. High-molecular-weight DNA was recovered with the Nanobind Plant Nuclei Kit (Circulomics; SKU NB-900-801-01), and needle-sheared 1x (FINE-JECT® 26Gx1” 0.45x25mm, LOT 14-13651). A 35-kb template library was prepared with the SMRTbell® Express Template Preparation Kit 2.0, and size-selected with the BluePippin system according to the manufacturer’s instructions (P/N 101-693-800-01, Pacific Biosciences, California, USA). In addition, a PCR-free library was prepared with the NxSeq® AmpFREE Low DNA Library Kit from Lucigen® according to the manufacturer’s instructions (Cat No. 14000-2). The final library was sequenced on a Pacific Biosciences Sequel instrument with Binding Kit 3.0. PacBio long-reads were assembled with Canu (v1.71) (Koren et al. 2017). The resulting contigs were first polished using the long-reads with the Arrow algorithm (v2.3.2; https://github.com/PacificBiosciences/GenomicConsensus), followed by a second polishing step with PCR-free short-reads using the Pilon algorithm (v1.22) (Walker et al. 2014). Lastly, the resulting contigs were scaffolded based on TAIR10 assembly by REVEAL (v0.2.1) (Linthorst et al. 2015). The previously generated Cdm-0 transcriptome sequencing data were mapped against the scaffolded genome assembly using HISAT (v2.0.5) (Kim, Langmead, and Salzberg 2015). Subsequently, the mapping results were used as extrinsic RNA sequencing evidence when annotating the genome using AUGUSTUS (v3.2.3) (Stanke et al. 2006). Transposable elements and repetitive regions were identified and masked prior to gene annotation using RepeatModeler2 (v2.01) (Flynn et al. 2020). Orthologous genes shared between Cdm-0 and the current A. thaliana reference annotation from Araport11 were identified using Orthofinder (v2.4.0) (Emms and Kelly 2019).

**Manual NLR annotation of the DM11 mapping interval**

The 20-25 Mb region of chromosome 1 was extracted from the Cdm-0 assembly. The assembly was used as a query against a subject FASTA file containing 167 NLR genes from the Col-0 reference accession using blastn (Altschul et al. 1990). Hits were binned in 20 kb intervals and the percentage identity between the queries and the subject was visualized across all bins. NLRs between At1g56510 to At1g64070 in Col-0 found in this interval were manually annotated based on the percentage identity plotted and on AUGUSTUS gene predictions (v2.5.5) (Stanke et al. 2006).

**GWAS**
Cdm-0-dependent hybrid necrosis in the F₁ progeny from crosses with 80 accessions (Chae et al. 2014) was scored as 0 or 1. The binary trait with accession information was submitted to the easyGWAS platform (Grimm et al. 2016) using the FaSTLMM algorithm. A -log₁₀(p value) was calculated for every SNP along the five A. thaliana chromosomes.

**Constructs and transgenic lines**

Genomic fragments were PCR amplified, cloned into pGEM®-T Easy (Promega, Madison, WI, USA) and then transferred to the binary vectors pMLBart, pCambia1300 or pFK210. Constructs were introduced into plants using Agrobacterium-mediated transformation (Weigel and Glazebrook 2002). T₁ transformants were selected on BASTA (pMLBart and pFK210) and crossed to incompatible accessions. Ethanol-inducible constructs were PCR amplified, cloned into pGEM®-T Easy, as part of a separate experiment, 2xHA tags were added via PCR and the whole fragment, which was then transferred to the pCR8® entry vector (ThermoFisher Scientific). Next, the genomic fragment was moved to the destination vector pZZ006 (Caddick et al. 1998) through the Gateway® LR reaction (ThermoFisher Scientific). Quality control for all constructs was done by Sanger sequencing. For transient expression in N. benthamiana, A. tumefaciens strains ASE (RLM1) or GV3101 (DM10 and At1g57650) were grown to an OD₆₀₀ of 1.2-1.8 and incubated in induction medium (10 mM MES (pH 5.6), 10 mM MgCl₂, and 150 µM acetosyringone) overnight. The cell suspensions were normalized to an OD₆₀₀ of 0.8 and co-infiltrations suspensions were mixed 1:1. Suspensions were then infiltrated into the abaxial side of N. benthamiana leaves. In the case of EtOH inducible constructs, infiltrated N. benthamiana were induced at 18 h post-infiltration (hpi) by irrigation with 1% ethanol and kept within a transparent plastic dome for another 18 h. DM10 N. benthamiana constructs shown in Fig 4 were co-expressed with a 35S::GFP construct as part of a larger experiment to test for candidate DM11 loci.

**Population genetic analyses**

Amino acid sequence conservation scores were calculated with ConSurf (Ashkenazy et al. 2016; Armon, Graur, and Ben-Tal 2001). SNPs occurring in repetitive regions and only present in one of the 73 extracted DM10 alleles were considered sequencing errors and were manually curated. Protein domains were predicted using InterProScan (Jones et al. 2014). LRR domains were predicted with LRRsearch and the score threshold was set at 7 (Bej et al. 2014). NLR motifs were defined based on previous studies (Meyers et al. 2003; Shao et al. 2016). Nonsynonymous to synonymous substitution rates were calculated using KaKs_Calculator (v2.0) (Wang et al. 2010) with the NG method (Nei and Gojobori 1986); a window length of 60 bp and a step size of 6 bp were chosen. Genomic regions of interest were subsetted from a 1135 genomes VCF file (1001 Genomes Consortium 2016) using VCFtools (v0.1.14) (Danecek et al. 2011). The resulting VCF file was filtered by MAF=0.01 and a maximum percent of missing data per SNP of 30%. Sequences were converted to FASTA,
aligned with MUSCLE (v3.8.31) (Edgar 2004) and then visualized with Aliview (v1.18.1) (Larsson 2014). Neighbor-Joining trees were calculated with Fastphylo (v1.0.1) (Khan et al. 2013) and visualized with iTol (Letunic and Bork 2007) (https://itol.embl.de/shared/cbarragan). Maximum-likelihood trees were calculated with RaxML (v0.6.0) using the GTR+G4 model (Stamatakis 2014). Linkage disequilibrium (r²), was calculated with PLINK (v1.90) (Purcell et al. 2007). Principal component analyses were calculated with smartPCA (Patterson, Price, and Reich 2006). Tajima’s D, FST, and nucleotide diversity (π) were also calculated with VCFtools. Maps were created with the R-packages maps (v3.3) and ggmap (v3.0) (Kahle and Wickham 2013). Admixture groups were assigned to each accession in accordance with the 1001 Genomes project (1001 Genomes Consortium 2016); since TueScha-9 had not been part of that study, admixture group assignments for it were estimated based on the genetic make-up of neighboring accessions. RLM1 homologs in A. lyrata and B. rapa were identified using the Ensembl Plants portal (Bolser et al. 2016). Sequences from the genome assemblies TAIR10 (A. thaliana), B. rapa (v1.5) and A. lyrata (v1.0) were used for phylogenetic analyses.

Data Availability
Sequencing data can be found at the European Nucleotide Archive (ENA) under project numbers PRJEB38267 (RNA-seq experiment) and PRJEB40125 (Cdm-0 assembly) and in the GenBank under accession numbers MT488482 to MT488554 (DM10 alleles).

Author contributions
Conceptualization: ACB, DW, EC.
Formal analysis: ACB, MC, RL, FR, HA, EC.
Funding acquisition: DW, EC.
Investigation: ACB, JW, WYC, EC.
Methodology: ACB, EC.
Project administration: DW.
Supervision: DW.
Writing – original draft: ACB.
Writing – review & editing: ACB, DW, EC.

Competing interests
The authors have declared that no competing interests exist.
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