Maize Plants Chimeric for an Autoactive Resistance Gene Display a Cell-Autonomous Hypersensitive Response but Non–Cell Autonomous Defense Signaling

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The maize gene *Rp1-D21* is a mutant form of the gene *Rp1-D* that confers resistance to common rust. *Rp1-D21* triggers a spontaneous defense response that occurs in the absence of the pathogen and includes a programmed cell death called the hypersensitive response (HR). Eleven plants heterozygous for *Rp1-D21*, in four different genetic backgrounds, were identified that had chimeric leaves with lesioned sectors showing HR abutting green nonlesioned sectors lacking HR. The *Rp1-D21* sequence derived from each of the lesioned portions of leaves was unaltered from the expected sequence whereas the *Rp1-D21* sequences from nine of the nonlesioned sectors displayed various mutations, and we were unable to amplify *Rp1-D21* from the other two nonlesioned sectors. In every case, the borders between the sectors were sharp, with no transition zone, suggesting that HR and chlorosis associated with *Rp1-D21* activity was cell autonomous. Expression of defense response marker genes was assessed in the lesioned and nonlesioned sectors as well as in near-isogenic plants lacking and carrying *Rp1-D21*. Defense gene expression was somewhat elevated in nonlesioned sectors abutting sectors carrying *Rp1-D21* compared with near-isogenic plants lacking *Rp1-D21*. This suggests that, whereas the HR itself was cell autonomous, other aspects of the defense response initiated by *Rp1-D21* were not.

Keywords: cell death, plant responses to pathogens, resistance genes

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The hypersensitive response (HR) is a rapid localized cell death in response to pathogen detection (Balint-Kurti 2019; Mur et al. 2008). It provides effective resistance to a wide range of biotrophic pathogens, including fungi, oomycetes, bacteria, and viruses, as well as other organisms such as insects (Rossi et al. 1998) and nematodes (Dropkin 1969) that form sustained intimate interactions with the host plant. It is generally less effective against, and may actually be beneficial to, necrotrophs, which require dead host tissue to complete their life cycle (Virdi et al. 2016).

The term HR has been used to describe a number of situations involving localized necrosis (Balint-Kurti 2019). Typically, HR is closely associated with gene-for-gene interactions (Flor 1942), in which a dominant resistance (*R*) gene in the host corresponds with a dominant avirulence (*Avr*) gene in the pathogen. Resistance, usually involving HR, is only conferred if both the *R* gene and the corresponding *Avr* gene are present in the same interaction. *Avr* genes are now understood to encode effectors: molecules secreted by the pathogen into the host to enable the pathogenesis process (Hogenhout et al. 2009). *Avr* proteins are simply effectors whose presence can be detected by specific *R*-proteins leading to the activation of the defense response.

Most *R* genes encode cytoplasmic-located proteins possessing both a nucleotide-binding site (NBS) or nucleotide-binding adaptor shared by APAF-1, *R* proteins, and CED-4 (NB-ARC) and leucine-rich (LRR) repeat domains known as NBS-LRR proteins (NLRs). Most NLRs carry either coiled-coil (CC) or toll-interleukin receptor (TIR) domains at their N terminus. Although similarities in NLR structure and function suggest a high level of conservation of mechanism, this is not necessarily the case. Notably, the mechanisms of their activation vary considerably (Kourelis and van der Hoon 2018; Wang and Balint-Kurti 2015). In general, the N-terminal domains are responsible for activating cell death pathways while the NBS and LRR domains regulate the activity of the NLR and auto-inhibit activation through intra-protein interactions (Kourelis and van der Hoon 2018; Wang et al. 2015b). NLR activity is additionally inhibited via a range of interactions with other proteins (Balint-Kurti 2019).

In a recent groundbreaking study, Wang et al. (2019a and b) demonstrated that, upon activation via indirect interaction with its cognate *Avr* protein, the *Arabidopsis thaliana* CC-NLR ZAR1 forms a homopentamer which relocates to the plasma membrane. Structural modeling suggested that the CC domains of the five subunits form a funnel-shaped structure which may be capable of creating pores in the plasma membrane to initiate the defense

response and HR. In other work, two TIR-NLRs, RPP1 and ROQ1 from *Arabidopsis* and *Nicotiana benthamiana*, respectively, were shown to form tetrameric structures upon direct binding of their cognate effectors (Ma et al. 2020; Martin et al. 2020; Tian and Li 2020). These findings, that activation of NLRs involve oligomerization into a so-called “resistosome”, are likely to be broadly applicable (Dangl and Jones 2019) but it is still not clear how these NLRs activate the defense response or if they all do so in a similar manner. Signal transduction is thought to involve the N-terminal domains. Structural modeling suggested that the CC domains of the five subunits form a funnel-shaped structure which may be capable of creating pores in the plasma membrane to initiate the defense response and HR (Wang et al. 2019a and b). TIR domains of NLRs were shown to possess an NAD⁺-cleaving activity required for cell death (Horsefield et al. 2019; Wan et al. 2019). In some cases, NLRs translocate to the nucleus upon activation and interact with transcription factors (Chang et al. 2013).

The *rp1* locus is a complex locus in the maize genome carrying multiple tandemly repeated CC-NLR paralogs that confer resistance to specific races of the fungus *Puccinia sorghi*, the causal agent of common rust (Hulbert 1997). Diverse *rp1* haplotypes have been identified and at least 16 distinct race-specific resistances have been mapped to this locus (Hulbert 1997). The *rp1* locus is notoriously unstable, and unequal crossing over between CC-NLR genes within *rp1* (Hulbert and Bennetzen 1991; Sudupak et al. 1993) sometimes results in intragenic recombination (i.e., the recombination of the 5' end of one paralog with the 3' end of another, to produce a chimeric gene). *Rp1-D21* is one such allele that resulted from intragenic recombination between the *Rp1* paralogs *Rp1-D* and *Rp1-dp2*. The protein encoded by *Rp1-D21* is spontaneously activated without the need for pathogen recognition and causes HR lesions on leaves of maize plants in the absence of pathogen (Hu et al. 1996; Wang et al. 2015b). The severity of the HR phenotype conferred by *Rp1-D21*, and the associated stunting of growth, is genetic background dependent (Chintamanani et al. 2010; Negeri et al. 2013).

The mechanism of activation of *Rp1-D21*, the loci affecting the severity of the HR phenotype it confers, and the proteins and pathways it interacts with have been the subject of a number of studies (Chaikam et al. 2011; Chintamanani et al. 2010; He et al. 2019a and b; Murphree et al. 2020; Negeri et al. 2013; Olukolu et al. 2013, 2014; Wang and Balint-Kurti 2016, 2015; Wang et al. 2015a and b), and several of the mechanisms by which *Rp1-D21* is regulated have been partially elucidated. However, using this system, it has not been possible to unambiguously investigate the relationships between cells in which *Rp1-D21* is activated and those in which it is not.

Chimeric, or genetic mosaic, plants have been used extensively in the study of plant development (Freeling 1992; Scanlon and Freeling 1997). These plants carry juxtaposed sectors that differ for one or more genes, and this allows the investigation of the cell-autonomous nature of the action of these genes and their role in signaling between cells (Fyvie et al. 2000). The only reported use of genetic mosaics to investigate R-protein action involved *Rp1*. Bennetzen et al. (1988) used X-ray treatment of seed heterozygous for *Rp1* to generate leaf sectors with chromosome breaks on the short arm of chromosome 10. These sectors lacked the *oil yellow* (*Oy*) gene which caused the sectors to be yellow. The yellow sectors were also predicted to lack *Rp1* because *Rp1* is distal to *Oy* on chromosome 10. *P. sorghi* was able to infect the yellow sectors and produce pustules, whereas infection of green parts of the plant resulted in HR. This occurred even in cases where the yellow sectors were only six cells wide. HR initiated in green sectors that bordered yellow sectors appeared to propagate a short distance into the yellow sectors but propagated much more extensively in the green sectors. Overall, the authors stated that these results demonstrated that “the initiation of the hypersensitive

response and resistance to *P. sorghi* require a cell-autonomous non-diffusible factor specified by *Rp1*”. They also reported that the *Rp1* gene was likely involved in “propagation as well as initiation of the necrotic response”.

Jones and Deverall (1978) used a temperature sensitive R protein and a “leaf transplant” technique to mechanically create wheat genetic mosaics to investigate the cell autonomy of HR. The epidermis of infected and uninfected wheat leaves was peeled away, and the mesophyll cell layers of both leaves were then brought into contact and held together for several days. By using cultivars with and without the *Lr20 P. recondita* R gene, they were able to show that necrosis (presumably caused by HR) occurred in uninfected tissue carrying *Lr20* when it was brought into contact with tissue infected with *P. recondita* that was avirulent on cultivars carrying *Lr20*. The genotype of the infected tissue did not affect the presence or absence of necrosis in the uninfected tissue. This suggested that *Lr20* gene action was cell autonomous because the presence of *Lr20* in the infected tissue was not enough to induce HR in the uninfected tissue. It also suggested that *Lr20* was required for the propagation of the HR. Although the molecular identity of the *Lr20* gene is not yet described, it is likely to be an NLR because it is a dominant gene (Browder 1972) conferring disease resistance associated with an HR.

Therefore, the findings of both the Jones and Deverall (1978) and the Bennetzen et al. (1988) studies are largely similar in suggesting that R gene action is largely cell autonomous and that R genes are required for the activation and the propagation of a HR. In this study, we were able to use updated techniques to confirm and extend these findings. We used spontaneously occurring and ethyl methanesulfonate (EMS)-generated chimeric plants to show that the HR induced by *Rp1-D21* is cell autonomous, though some aspects of the defense response signaling are not. We identified a set of mutations in the *Rp1-D21* gene capable of suppressing or abrogating activity and provide evidence that *Rp1-D21* is activated in most cells in which it is present, not only the cells displaying HR cell death.

RESULTS AND DISCUSSION

Identification of plants with chimeric leaves suggests that the HR phenotype conferred by *Rp1-D21* is cell autonomous.

Approximately 50,000 plants carrying *Rp1-D21* were observed in the summer of 2017, as part of a related study. Among these, two chimeric plants carrying leaf sectors of nonlesioned tissue abutting tissue displaying HR-induced lesions were identified. Chimeric mutant 1 (CM1) was derived from a Tx303 × H95:Rp1-D21 cross and chimeric mutant 2 (CM2) was derived from a M37W × H95:Rp1-D21 cross (Fig. 1A). These chimeras apparently resulted from spontaneous inactivation of *Rp1-D21*.

Two independent attempts were made to develop more chimeric mutant plants displaying HR-induced lesions in different maize genetic background. The *Rp1-D21#4* allele is a previously described weak allele of *Rp1-D21* derived from an EMS mutagenesis screen (Wang et al. 2015b) of previously unknown molecular nature. The B73:Rp1-D21#4 line is the maize line B73 carrying an introgression of *Rp1-D21#4* maintained in a heterozygous state. EMS mutagenesis of 500 B73:Rp1-D21#4 seed yielded 11 chimeric plants displaying sectors of nonlesioned tissue on their leaves. Three of these plants had sectors large enough to isolate tissue sufficient for molecular analysis. These plants were referred as chimeric mutants 3-1, 3-2, and 3-3 (CM3-1, CM3-2, and CM3-3) (Fig. 1A). The H95:Rp1-D21 line is the maize line H95 carrying an introgression of *Rp1-D21* maintained in a heterozygous state. Among 1,100 EMS mutagenized H95:Rp1-D21 seeds, 18 chimeric plants displaying nonlesioned sectors were identified. Six of these plants showing the

clearest sectors were selected for further study. These plants were named chimeric mutants 4-1, 4-5, 4-6, 4-10, 4-12, and 4-13 (CM4-1, CM4-5, CM4-6, CM4-10, CM4-12, and CM4-13).

In every case, regardless of size or orientation, the border between the lesioned and nonlesioned tissue was abrupt, with no transition zone, and this was apparent at both the macroscopic and microscopic levels (Fig. 1A and B), suggestive of a cell-autonomous phenotype. All patterns were consistent with mitotic transmission of the sector. Also notable was the fact that many living (nonbrown) cells in the lesioned sector were significantly lighter green or chlorotic compared with the nonlesioned sector. This chlorosis was also entirely confined to the lesioned sector with a clear demarcation at the sector boundary, indicating that the chlorosis phenotype was cell autonomous also (Fig. 1A, magnified insets). The fact the cells close to the border in the nonlesioned sectors did not display any chlorosis strongly suggests that the chlorosis observed in the lesioned sector is due to the effects of *Rp1-D21* in those cells rather than the fact that they are adjacent to dead cells. This suggests that *Rp1-D21* is activated in all or most of the cells in the lesioned sectors. Within the lesioned areas, cells varied from light green to yellow to brown and necrotic. The patches of light-green cells in the lesioned sectors may indicate areas in *Rp1-D21* has not yet been activated or has only recently activated, or where negative regulation of HR has maintained viability.

Analysis of cell death marker gene expression.

Cell death marker genes such as the *hairpin-induced gene* (*ZmHin1*) and *EF-hand Ca²⁺-binding protein* (*ZmCCD1*)

(Pontier et al. 1999; Takezawa 2000) have been shown to be induced during HR cell death in several studies (Christensen et al. 2015; Yeom et al. 2012). Quantitative real-time PCR (qRT-PCR) analysis of CM1, CM2, and CM3 plants showed that the transcript levels of *ZmHin1* and *ZmCCD1* were significantly higher in lesioned sectors and nonchimeric isogenic lesioned plants carrying *Rp1-D21* than in nonlesioned sectors and wild-type isogenic plants lacking the *Rp1-D21* gene, here referred to as nonlesioned plants (Fig. 2). No increased expression was observed in nonlesioned sectors compared with nonlesioned plants. In the case of *ZmHin1*, we were unable to amplify the gene from either lesioned or nonlesioned sectors of CM3 plants. We suspect that this may be associated with the age of the plants when sampled (70 days after planting for CM1 and CM2 and 28 days for CM3). These results support the conclusion that the HR induced by *Rp1-D21* is a cell-autonomous phenotype.

Spontaneous mutations in *Rp1-D21* gene led to formation of nonlesioned chimeric sectors and confirm cell-autonomous action of *Rp1-D21*.

We attempted to amplify and sequence the *Rp1-D21* gene from lesioned and nonlesioned sectors of the 11 chimeric mutants using both Wideseq and Sanger sequencing approaches (Table 1). As expected, in all of the lesioned sectors from CM1, CM2, and CM4, the *Rp1-D21* (Wang et al. 2015b) sequence was recovered. The lesioned sectors of CM3-1, CM3-2, and CM3-3 carried the *Rp1-D21#4* allele, which we here identify for the first time as an A₃₆₁₄T single-nucleotide polymorphism

Fig. 1. Clear demarcation between lesioned and nonlesioned sectors in the chimeric *Rp1-D21* plants. **A**, Macroscopic images of chimeric mutant (CM) sectors showing clear demarcation between lesioned and nonlesioned sectors. Representative CM3 and CM4 leaves are shown. **B**, Microscopic image of boundary between lesioned and nonlesioned sectors of a representative chimeric mutant showing clear demarcation between sectors.

(SNP) relative to *Rp1-D21* corresponding to an M1205K polymorphism at the protein level (Fig. 3; Supplementary Fig. S2). This is the only missense mutation and the likely cause of the reduced severity of the *Rp1-D21#4* mutant.

The *Rp1-D21* sequence derived from the nonlesioned sectors of CM1 contained novel alleles of encoded an A₃₁₅₂T SNP relative to *Rp1-D21*, which is predicted to cause a V₁₀₅₁D missense mutation. The CM3-1 nonlesioned sector had a G to A transition relative to *Rp1-D21#4* at position 244, resulting in a missense mutation encoding the change D82N in the resulting protein (Fig. 3; Supplementary Fig. S3) that alters a residue in the conserved EDVID motif in the CC domain of *Rp1-D21#4*. This same change was previously recovered as a germinal suppressor of HR caused by *Rp1-D21* (Wang et al. 2015b). The CM3-3 nonlesioned sector had a G to A transition in *Rp1-D21#4* at position 3,013, resulting in an A1005T change within the LRR domain of *Rp1-D21* (Fig. 3; Supplementary Fig. S3).

Wideseq results suggested that the *Rp1-D21* allele in the CM2 nonlesioned sectors had a C₆₈₀A substitution in the *Rp1-D21* sequence predicted to cause T₂₂₇N missense mutation, and a large deletion in the LRR domain. However, we were

unable to confirm this mutation using a Sanger sequencing approach despite numerous attempts. Similarly, Wideseq data suggested that the CM3-2 nonlesioned sector carried a C to T

Table 1. Mutations identified in nonlesioned sectors of chimeric plants^a

Plant	Single-nucleotide polymorphism	Wideseq sequences with mutation (%)
CM1	A ₃₁₅₂ T	99.8
CM3-1	G ₂₄₄ A	51.0
CM3-3	G ₃₀₁₃ A	94.5
CM4-1	T ₃₅₇₄ C	—
CM4-5	Deletion of T at 3557	—
CM4-6	C ₁₉₀₃ T, A ₂₅₀₇ G	—
CM4-10	A ₅₈₇ G	—
CM4-12	G ₂₂₆₃ A	—
CM4-13	A ₂₁₅₅ G, A ₂₄₅₃ G	—

^a Mutations in chimeric mutant 2 (CM2) and CM3-2 were not verified by Sanger sequencing and, therefore, are not shown in the table. Mutations in CM1, CM3-1, and CM3-3 were identified by Wideseq and Sanger sequencing. For these mutations, the percentages of sequences from nonlesioned sectors carrying mutations are shown.

Fig. 2. Expression analysis of cell death marker genes. Quantitative reverse-transcription (qRT)-PCR of cell death marker genes *ZmCCD1* and *ZmHin1* in nonsectored leaves of plants carrying *Rp1-D21* (mutant) and in lesioned sectors of chimeric mutant 1 (CM1), CM2, and CM3. Histogram bars are color coded to indicate type of tissue. A visual key is shown on the right. Results displayed are the averages of all biological replicates in each case. For CM1 and CM2, three biological replicates we collected as individual leaves from the same plant as described. For CM3 qRT-PCR results, we considered samples from CM3-1, CM3-2, and CM3-3 to be three biological replicates. The error bar shows standard error. Significance between treatments were analyzed using Student's *t* test (asterisks [* , ** , ***] indicate *P* < 0.05, 0.01, and 0.001, respectively).

transition relative to *Rp1-D21#4* at position 2,308 encoding a change of P770S in the LRR domain. However, as with the CM2 nonlesioned sector, we were unable to verify this mutation using Sanger sequencing. The Wideseq approach relies on the assembly of multiple short sequences generated using MiSeq. Because the *rp1* locus is complex, carrying multiple similar homologous sequences, an approach of this type may result in the misassembly of sequences from different homologs. For this reason, in Table 1 and Fig. 3, we are only reporting sequences verified using a Sanger sequencing approach. Nevertheless, we are confident that the *Rp1-D21* gene is mutated and nonfunctional in the CM2 and CM3-2 nonlesioned sector due both to the clear visible phenotype and the fact that we also did not detect “wild-type” *Rp1-D21* or *Rp1-D21#4* sequences as we did in all of the lesioned sectors of all 11 chimeric plants investigated.

The *Rp1-D21* sequence from the CM4-1 nonlesioned sector had a T to C transition at position 3,574, resulting in a P1192S amino acid change in the LRR domain of *Rp1-D21*. The nonlesioned CM4-5 sector had a deletion of T at position 3,556 leading to a frame-shift mutation, resulting in a truncated protein. The CM4-6 nonlesioned sector carried two missense mutations in the

LRR domain of *Rp1-D21* gene. The first was a C to T transition at position 1,903, resulting in an F635L at the protein level. The second was an A to G transition at position 2,507, causing a G836E amino acid change. The *Rp1-D21* sequence derived from nonlesioned sector of CM4-10 had an A to G transition at position 587 encoding a G196D change in the NB-ARC domain close to the border between CC and NB-ARC domains. The CM4-12 nonlesioned sector had a G to A transition at position 2,263 which led to change in K755E in the LRR domain. The CM4-13 nonlesioned sector carried two missense mutations in the LRR domain. The first was at position 2,155, where an A to G transition resulted in change of G719S at the protein level, and the second was at position 2,453, where an A to G transition led to a G818E change (Fig. 3). Detailed sequence alignments are shown in Supplementary Figures S1 to S4.

The facts that all of the lesioned sectors carried “normal” *Rp1-D21* or *Rp1-D21#4* sequences; that, in nine of the nonlesioned sectors, we identified mutated versions of *Rp1-D21* or *Rp1-D21#4*; and that, in the other two nonlesioned sectors, we did not identify *Rp1-D21* or *Rp1-D21#4* sequences; together with the observation that the sector boundaries are abrupt (Fig. 1), confirm that the HR induced by *Rp1-D21* is cell autonomous.

The CM1 and CM2 sectors were caused by spontaneous mutations while the nine CM3 and CM4 sectors were identified on plants treated with the EMS mutagen. The large majority of mutations caused by EMS are G/C to A/T (Kim et al. 2006). Therefore, it is notable that 6 of the 10 mutations we observed in the CM3 and CM4 nonlesioned sectors were not the expected G/C to A/T transitions. It has been noted previously that the *Rp1* locus is highly unstable due to its complex nature, with frequent mutations associated with meiotic unequal crossover events (Sudupak et al. 1993); thus, some of the observed *Rp1-D21* loss-of-function mutations may not have been related to EMS. EMS can also induce double strand breaks (Ma et al. 2011; Sega 1984; Wagner et al. 2003), which may be repaired by a combination of base excision repair and homologous repair (Klapacz et al. 2016), and it can also induce gross chromosomal abnormalities (Sato and Gaul 1967). It is likely that in the context of the complex tandemly repeated *rp1* locus, double strand breaks caused by EMS may be repaired using paralogous sequences as templates, inducing more complex mutations as well as simple point mutations in somatic cells.

Previously, Wang et al. (2015b) had identified 12 missense and two nonsense mutations in *Rp1-D21* that abrogated the spontaneous HR phenotype. The 12 missense mutants were distributed among the domains: five in the CC, three in the NB-ARC, and four in the LRR domain. Of the 11 mutations identified here, nine were in the LRR, one in the CC domain, and one in the NB-ARC domain. Although the LRR is the largest domain and, therefore, the most likely to be mutated by chance, this distribution is a little surprising. The CC domain is responsible for causing HR while the NB and LRR domains play roles in activation and intraprotein interactions that inhibit activity (Wang et al. 2015b); thus, it would have seemed likely more of mutations suppressing HR would occur in the CC domain.

The maize leaf develops as five tissue layers: two outer epidermal cell layers, a central layer, and two layers in between these (Freeling 1992). As the leaf develops, the middle three layers intermingle so that three developmental layers can be distinguished (Freeling 1992). Depending on the precise position of the cell carrying the original mutation in the meristem, chimeric leaf sectors may carry a mutation only in certain tissue layers or in all of them (Becraft et al. 1990; Freeling 1992; Scanlon and Freeling 1997). The Wideseq technique used to sequence *Rp1-D21* from the CM1, CM2, and CM3 nonlesioned sectors provided high sequence coverage (189 to 4,847x) (Supplementary Table S2). In order to determine the structures of the chimeras in this study, we

Fig. 3. Mutations causing loss of hypersensitive response (HR) phenotype in nonlesioned sectors. Three domains of the *Rp1-D21* protein—the coiled-coil domain; nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4 domain; and leucine-rich repeat—are indicated by blue, orange, and green shading, respectively. Predicted amino acid polymorphisms identified are denoted below each sequence. *Rp1-D21* sequences from chimeric mutant 1 (CM1), CM4-1, CM4-5, CM4-6, CM4-10, CM4-12, and CM4-13 nonlesioned sectors are compared with the reported *Rp1-D21* sequence while *Rp1D21#4* is used as reference to compare sequences derived from CM3-1 and CM3-3 nonlesioned sectors. Only sequences verified by Sanger sequencing are shown. One asterisk (*) = previously shown by Wang et al. (2015b) and two (**) = transient expression in *N. benthamiana* confirmed no HR induced.

analyzed the sequencing data derived from each sector to determine the proportion of sequences carrying the mutated allele. If a significant proportion of the sequences from the nonlesioned sectors carried the original allele, that would indicate that the sector was composed of genetically distinct layers. Of the three mutations identified by Wideseq and confirmed by Sanger sequencing shown in Table 1, two were found in more than 90% of the sequences (Table 1), indicating that they are likely present in all developmental tissue layers. By contrast, the G₂₄₄A from CM3-1 was identified in 51% of the sequences, suggesting that it is likely they are not present in all tissue layers.

Ectopic expression of the CM3-3 *Rp1-D21* allele does not induce cell death.

To validate the effect of one of the mutations identified in the *Rp1-D21* gene, a transient expression study in *N. benthamiana* was performed. We have previously shown that the HR phenotype conferred by *Rp1-D21* in maize can be effectively recapitulated in this system (Wang et al. 2015b). Phenotypic observations at 72 h postinoculation (hpi) (Fig. 4A) shows that the *Rp1-D21* protein encoded by the CM3-3 allele failed to induce HR whereas the functional *Rp1-D21* protein derived induced HR as expected. Furthermore, ion leakage analysis confirms this result (Fig. 4B).

Fig. 4. Transient expression of *Rp1-D21* from chimeric mutant (CM) CM3-3 in *Nicotiana benthamiana*. **A**, *N. benthamiana* leaf showing cell death caused by expression of the functional *Rp1-D21* allele compared with the mutated CM3-3 *Rp1-D21* allele whose expression does not induce cell death. EV = empty vector control **B**, Ion leakage analysis shows lower conductivity ratio (C1 = initial conductivity/C2 = final conductivity), indicating less cell death from tissue in which CM3-3 *Rp1-D21* allele was expressed compared with leaf tissue in which the functional *Rp1-D21* clone was transiently expressed. **C**, Western blot analysis using anti-HA antibody shows that both *Rp1-D21* proteins are expressed in the *N. benthamiana* heterologous system. Significance between treatments were analyzed using one-way analysis of variance: Three asterisks (***) indicate $P < 0.001$. This experiment was repeated three times with similar results.

Protein analysis showed that each protein was successfully expressed (Fig. 4C).

HR-induced cell death is cell autonomous but some defense response transcript levels respond to a diffusible signal.

In order to look at other aspects of the *Rp1-D21* phenotype, transcript levels of various markers of disease response were measured in the lesioned and nonlesioned sectors of CM1, CM2, and CM3 plants. In addition to the sectors, transcript abundance was also determined in isogenic *Rp1-D21* and wild-type control plants grown under the same conditions and at the same time as the chimeric plants in neighboring rows or pots. HR is typically associated with a wider defense response that includes the upregulation of a suite of pathogenesis related (PR) genes (Ali et al. 2018). We were interested to determine whether the induction of PR gene expression was cell autonomous in a similar way to the HR, chlorosis and cell death-marker gene expression phenotypes.

As described above for the marker genes associated with cell death, we determined the transcript levels of three genes—*pathogenesis-related 1* (*ZmPR1*), *pathogenesis-related 4b* (*ZmPR4b*), and *pathogenesis-related protein 5* (*ZmPR5*)—in the lesioned and nonlesioned sectors of CM1, CM2, and CM3 and their corresponding isogenic nonchimeric lesioned and nonlesioned counterparts (Fig. 5). *ZmPR1*, *ZmPR4b*, and *ZmPR5* have been previously shown to be induced during the defense response in maize (Chintamanani et al. 2010; Christensen et al. 2015) and *ZmPR1* and *ZmPR5* were previously reported to be salicylic acid induced (Morris et al. 1998; Vargas et al. 2012).

ZmPR1, *ZmPR4b*, and *ZmPR5* showed relatively higher expression levels in nonchimeric lesioned plants carrying the *Rp1-D21* gene and in lesioned sectors of CM1, CM2, and CM3 chimeric plants than in their nonlesioned nonchimeric

counterparts (Fig. 5). Of particular interest was the observation that expression levels of *ZmPR4b* and *ZmPR5* were significantly higher in nonlesioned sectors of CM1 and CM2 chimeric plants compared with their nonlesioned nonchimeric counterparts, though this was not the case for CM3 (Fig. 5). The expression of *ZmPR1* in the nonlesioned sectors of CM1 was significantly higher than in its nonlesioned nonchimeric counterpart but this was not the case for CM2 or CM3.

We also assessed the expression of *ZmCCoAOMT2* (Fig. 5), which encodes a member of the family of enzymes that includes lignin biosynthetic enzyme caffeoyl-coenzyme A (CoA) O-methyltransferase. A previous study (Wang and Balint-Kurti 2016) showed that *Rp1-D21*-induced HR could be suppressed by *ZmCCoAOMT2* and that *ZmCCoAOMT2* transcript levels increased due to HR induced by *Rp1-D21*. As expected, the *ZmCCoAOMT2* expression levels were significantly higher in all lesioned plants and sectors than in the nonlesioned controls (Fig. 5). In this case, expression in the nonlesioned sectors of CM2 (but not of CM1 and CM3) was significantly higher than in its nonlesioned nonchimeric counterpart.

Although not entirely consistent between plants and between genes, these results suggest that, whereas the HR and chlorosis phenotypes associated with *Rp1-D21* are cell autonomous, this is not true for some of the associated defense responses. It appears that a signal from the lesioned sector induces increased PR gene expression in the neighboring nonlesioned sectors. Although we have no direct evidence, it seems likely that this signal is salicylic acid or a close derivative. Betsuyaku et al. (2018) reported high expression of *PR1* and another defense marker gene, *VSP1*, in *Arabidopsis* leaves in the ring of cells surrounding cells undergoing an HR induced by the activation of the Rps2 NLR protein. They showed that these regions were relatively small (measuring less than 1 mm in width), that their borders were relatively well defined, and that each gene was

Fig. 5. Gene expression of defense-response marker genes in lesioned and nonlesioned sectors and plants. Quantitative reverse-transcription (qRT)-PCR of *ZmPR1*, *ZmPR4b*, *ZmPR5*, and *ZmCCoAOMT2* genes in leaves of plants carrying *Rp1-D21* (mutant); lesioned sectors of chimeric mutant 1 (CM1), CM2, and CM3 (lesioned sector) in wild-type plants lacking *Rp1-D21* (wild type) and in nonlesioned sectors of CM1, CM2, and CM3 (nonlesioned sector). Histogram bars are color coded to indicate type of tissue. A visual key is shown on the right. Results displayed are the averages of all biological replicates in each case. For CM1 and CM2, three biological replicates we collected as individual leaves from the same plant as described. For CM3 qRT-PCR results, we considered samples from CM3-1 to CM3-3 to be three biological replicates. The error bar shows standard error. Significance between treatments were analyzed using Student's *t* test; asterisks (*, **, or ***) indicate $P < 0.05$, 0.01, and 0.001, respectively.

expressed in a distinct spatiotemporal pattern. The data presented here are far less detailed with respect to patterns of gene expression but it should be noted that the levels of defense gene upregulation in the nonlesioned sectors might vary substantially, depending on how far they are from the lesioned sectors.

In summary, we suggest that cell death and chlorosis associated with the *Rp1-D21* phenotype is cell autonomous, while another aspect (induction of PR genes) is non-cell autonomous. Freeling (1992) suggested that “It is entirely expected for dominant phenotypes to be cell autonomous for one component of phenotype and non-cell autonomous for another” and demonstrated that this was true for the maize *D8* dwarf allele. Our results suggest that *Rp1-D21* is necessary and sufficient for the observed spontaneous HR phenotype and are consistent with previous reports (Bennetzen et al. 1988). The fact that macroscopic spots of cell death occur on leaves and leaf sectors carrying *Rp1-D21* suggests that a “pro-death” signal must be produced by cells undergoing HR that predisposes neighboring cells also to undergo HR. However, *Rp1-D21* is required to sensitize the neighboring cells so that they are competent to respond to this signal. In other systems, pathogen-induced HR can manifest as a single-cell phenotype in some cases or as macroscopic spots in others (Balint-Kurti 2019). Mutations affecting the autophagy system (Liu et al. 2005; Patel and Dinesh-Kumar 2008) or the production of reactive oxygen species (Dietrich et al. 1997) can lead to the unrestricted spread of cell death once initiated by activation of an NLR. Although HR is cell autonomous in this case, we should be cautious in assuming that this is universally true for HR initiated by all NLRs. The interaction between processes facilitating the spread of HR cell death, which may enhance resistance to biotrophic pathogens, and processes that contain it, which may reduce the negative growth aspects of HR, has not been clearly elucidated and is likely to be quite complex, reflecting aspects of the plant–pathogen evolutionary arms race.

MATERIALS AND METHODS

Genetic material and mutagenesis.

Eleven mutants, with leaves chimeric for *Rp1-D21* derived from four independent populations, were used for this study. CM1 was a spontaneous mutant identified in the field and was derived from a cross between the maize lines Tx303 and H95:Rp1-D21 (the maize line H95 carrying *Rp1-D21* in a heterozygous state). CM2 was a spontaneous mutant identified in the field and was derived from a cross between the maize lines M37 and H95:Rp1-D21. Both CM1 and CM2 were identified in the same field in Clayton, NC in summer 2017.

CM3-1, CM3-2, and CM3-3 were derived from a set of B73:Rp1-D21#4 plants (the maize line B73 carrying an allele of *Rp1-D21* called *Rp1-D21#4* in a heterozygous state), which were grown from seed mutagenized using EMS. The protocol for EMS seed mutagenesis was as follows: 500 seeds were soaked in 500 ml of 20 mM EMS for 6 h in darkness, then rinsed in running water for 8 h. The seed were dried and planted in a greenhouse at Purdue University (West Lafayette, IN, U.S.A.). The plants were grown at 25°C with a cycle of 16 h/day and 8 h/night. Among 500 plants grown in the greenhouse facility, three plants (CM3-1, CM3-2, and CM3-3) were identified with chimeric wild-type sectors in their leaves.

CM4-1, CM4-5, CM4-6, CM4-10, CM4-12, and CM4-13 were derived from a set of H95:Rp1-D21 plants (the maize line H95 carrying *Rp1-D21* in a heterozygous state), which were grown from seed mutagenized using EMS using the same protocol as described above. The seed were dried and planted in field at Clayton, NC in summer 2019. Among 1,100 plants, 18 plants showed chimeric wild-type sectors, among which six plants (CM4-1,

CM4-5, CM4-6, CM4-10, CM4-12, and CM4-13) displaying the clearest sectors were selected for further studies.

Sample collection.

Multiple leaves displayed *Rp1-D21* sectors on the CM1 and CM2 plants. For each plant, 5-cm² samples of tissue were excised from lesioned sectors and nonlesioned sectors. The closest edge of sampled tissue square was approximately 0.5 cm from the sector boundary in each case. Samples were collected from three individual leaves of the same plant and are considered as biological replicates. Because all of the leaves in the plant showed chimeric sectors, samples were collected from leaf four, the ear leaf, and the flag leaf. Similarly, leaf samples from leaf four, the ear leaf, and the flag leaf were collected from three different (nonchimeric) mutant and wild-type plants from Tx303 × H95:Rp1-D21 and M37W × H95:Rp1-D21 families and used as isogenic controls for CM1 and CM2, respectively. All of the samples were collected from 70-day-old plants.

Samples were collected from the lesioned and nonlesioned sectors of 4-week-old CM3-1, CM3-2, and CM3-3 plants, the three EMS-treated B73:Rp1-D21#4 plants displaying chimeric sectors. The near edge of the sampled tissue was approximately 2 mm away from the sector border in each case. The distance between borders is smaller in CM3 compared with CM1 and CM2, because the CM3 plants were younger and their leaves were considerably smaller. Additionally, samples were collected from three wild-type (B73) and three lesioned (B73:Rp1-D21#4) plants as controls. All samples were collected 4 weeks after planting, frozen in liquid nitrogen immediately, and stored at –80°C until further use.

Samples were collected from the lesioned and nonlesioned sectors of 7-week-old CM4-1, CM4-5, CM4-6, CM4-10, CM4-12, and CM4-13 plants, six EMS-treated H95:Rp1-D21 plants displaying chimeric sectors. For each plant, 5-cm² samples of tissue were excised from lesioned sectors and nonlesioned sectors. The closest edge of sampled tissue square was approximately 0.5 cm from the sector boundary in each case. All of the samples were collected, frozen in liquid nitrogen immediately, and stored at –80°C until further use.

Total RNA isolation and amplification of *Rp1-D21* coding sequence.

Ten 6-mm-diameter leaf punches from the samples described above were used to isolate RNA. Total RNA was extracted from maize leaf samples with an RNeasy Plant Mini Kit (Qiagen) and treated with RNase-free DNase I (Thermo Fisher Scientific) to remove residual genomic DNA, according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 3 µg of total RNA with RevertAid reverse transcription (Thermo Fisher Scientific) with OligodT primers, according to the manufacturer's protocol.

Primers Rp1D21F1 and Rp1D21R1 (Supplementary Table S1) were used to amplify the *Rp1-D21* coding sequence with Q5 high-fidelity DNA polymerase (M0491; New England Biolabs). The reaction mixture and conditions for PCR were followed according to the manufacturer's protocol. A second primer set (Rp1D21F2 and Rp1D21R2) (Supplementary Table S1) was used to amplify the *Rp1-D21* coding sequence with Q5 high-fidelity DNA polymerase (M0491; New England Biolabs). The product derived from the Rp1D21F2/R2 amplification was used exclusively for Sanger sequencing.

Both sets of primers that amplify *Rp1-D21* were validated using H95:Rp1-D21 and B73:Rp1-D21 as positive control templates and DNA from B73, Tx303, H95, and M37W as negative control templates.

Expression analysis using qRT-PCR.

Primers for qRT-PCR were first used to generate standard PCR products, which were then sequenced using standard Sanger sequencing to confirm that the expected gene fragments were amplified. qRT-PCR was carried out on a CFX384 real-time PCR detection system (Bio-Rad) with SsoAdvanced SYBR Green Master mix (Bio-Rad), according to the manufacturer's instructions. The primers used for qRT-PCR studies are provided in Supplementary Table S1. Gene expression was measured relative to the housekeeping gene *ZmGAPDH1*. The $2^{-\Delta\Delta CT}$ method (normalized to *ZmGAPDH1* expression and relative to a wild-type control) was used to calculate relative gene expression (Livak and Schmittgen 2001). Three technical replicates were performed for each sample. Three individual leaves in CM1 and CM2 were considered as three biological replicates. In the case of CM3, samples from CM3-1, CM3-2, and CM3-3 were considered as biological replicates. Statistical analysis was calculated using Student's *t* test (GraphPad PRISM).

Sequencing and assembly of *Rp1-D21* cDNA.

Two different approaches were used for sequencing. The *Rp1-D21* PCR products derived from amplification using the Rp1D21F1/R1 primers on DNA derived from CM1, CM2, CM3-1, CM3-2, and CM3-3 plants were purified using a DNA Clean & Concentrator-5 kit (Zymo Research). The purified PCR products were quantified using a Nanodrop 2000/2000c spectrophotometer (Thermo Fisher Scientific). The PCR amplicon was sequenced by a Wideseq methodology, as detailed by Khangura et al. (2019). Briefly, sequencing libraries were generated using a hyperactive variant of Tn5 transposase using the Illumina Nextera DNA library preparation kit. The multiplexed libraries were sequenced using the MiSeq instrument (Illumina) to generate the paired-end reads (2×250 bp) at the Purdue Genomics Core Facility. Paired-end reads from each sample were processed to remove adapters and poor-quality bases (<Phred-20) using Trimmomatic (Bolger et al. 2014) with the following settings: LEADING:20 TRAILING:20 MINLEN:30. Only the filtered paired reads were used for further processing. The filtered paired-end reads were aligned to the *Rp1-D21* reference mRNA sequence (NCBI accession number KF951062.1) using short-read aligner BWA version 0.7.12 (Li and Durbin 2009). The alignments were generated using the BWA sample command with default parameters to generate Sequence Alignment/Map (SAM) files. The SAM files were converted into Binary Alignment Map (BAM) files, followed by sorting, indexing, and finally variant calling using the mpileup function in SAMtools suite version 1.3 (Li et al. 2009). The detected variants were manually inspected by visualizing each sample in IGV version 2.8.2 (Robinson et al. 2011) using the sorted BAM files. Mean coverage across the reference-aligned reads for each sample were calculated using the depth function in SAMtools (Table 1; Supplementary Table S2). All derived sequences have been deposited in GenBank (Supplementary Table S3). Multiple sequence alignments were performed using MEGA software (Kumar et al. 2004).

The second sequencing approach, Sanger sequencing, was performed on the *Rp1-D21* PCR products derived from amplification using the Rp1D21F2/R2 primers. PCR products were purified using a DNA Clean & Concentrator-5 kit (Zymo Research) and quantified using a Nanodrop 2000/2000c spectrophotometer (Thermo Fisher Scientific). They were cloned into pGEM-T vector (Promega Corp.) following the manufacturer's protocol. Two independent colonies for each recombinant clone were Sanger sequenced. The full-length sequence (3,873 bp) was achieved using seven nested primers (Supplementary Table S1, primers 15 to 25). The multiple sequence alignments shown

in Supplementary Figures S1 to S4 were performed using Geneious Prime software.

Cloning and transient expression in *N. benthamiana*.

The purified PCR products of *Rp1-D21* amplified from CM3-3 and from "normal" Rp1-D21 plants were cloned into pCR8/GW/TOPO TA vector following the manufacturer's protocol (Thermo Fisher Scientific). The resultant ligation mixtures were transformed into *Escherichia coli*, and colony PCR was performed to identify positive clones. The positive recombinant clone for each gene was further subcloned into the pGWB614 vector carrying the 3xHA tag at the C terminal using the gateway cloning protocol (Thermo Fisher Scientific). The resulting recombinant clone was further transformed into *Agrobacterium tumefaciens* for downstream applications.

Wild-type *N. benthamiana* plants were grown in the laboratory at 25°C with a cycle of 16 h of light and 8 h of darkness. *A. tumefaciens* GV3101 (pMP90) carrying the *Rp1-D21* expression constructs in the binary vector was grown at 28°C overnight in 50 ml of Luria-Bertani media with appropriate antibiotics. The culture was centrifuged at 4,000 rpm for 30 min at 4°C. The pellet was resuspended in infiltration buffer diluted to a final concentration of optical density at 600 nm (OD_{600}) = 0.6 and was combined with a p19 (Lakatos et al. 2004) silencing suppressor construct at OD_{600} = 0.3. The resultant mixture was used for transient expression in *N. benthamiana*. The detailed procedures were performed according to Wang et al. (2015b).

Protein analysis and Ion leakage.

For protein expression analysis, at least three leaf discs (1.2 mm in diameter) from each leaf were collected at 30 hpi. Total protein was isolated using 300 μ l of protein extraction buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA [pH 8.0], 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 10 mM dithiothreitol, 40 mM MG132, and 1 $\times$ plant protein protease inhibitor mixture) (Sigma-Aldrich). Samples were centrifuged at 14,000 rpm for 10 min at 4°C, and 50 μ l of supernatant was mixed with 2 $\times$ Laemmli sample buffer at a 1:1 ratio. The mixture was boiled at 95°C for 5 min and samples were used for SDS-polyacrylamide gel electrophoresis. The protein gel was transferred to nitrocellulose membrane and was incubated with anti-HA antibody conjugated with horseradish peroxidase (HRP) at 1:350 dilution (Roche). The HRP signal was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

In total, five leaf discs (2 mm in diameter) from different plants were used for measuring ion leakage. The detailed procedures were performed according to Wang et al. (2015b). The ion leakage was calculated using the C1/C2 ratio (where C1 = initial conductivity and C2 = final conductivity). The statistical analysis was calculated using one-way analysis of variance (GraphPad PRISM).

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