The CC-NB-LRR-Type Rdg2a Resistance Gene Confers Immunity to the Seed-Borne Barley Leaf Stripe Pathogen in the Absence of Hypersensitive Cell Death

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Abstract

Background: Leaf stripe disease on barley (Hordeum vulgare) is caused by the seed-transmitted hemi-biotrophic fungus Pyrenophora graminea. Race-specific resistance to leaf stripe is controlled by two known Rdg (Resistance to Drechslera graminea) genes: the H. spontaneum-derived Rdg1a and Rdg2a, identified in H. vulgare. The aim of the present work was to isolate the Rdg2a leaf stripe resistance gene, to characterize the Rdg2a locus organization and evolution and to elucidate the histological bases of Rdg2a-based leaf stripe resistance.

Principal Findings: We describe here the positional cloning and functional characterization of the leaf stripe resistance gene Rdg2a. At the Rdg2a locus, three sequence-related coiled-coil, nucleotide-binding site, and leucine-rich repeat (CC-NB-LRR) encoding genes were identified. Sequence comparisons suggested that paralogs of this resistance locus evolved through recent gene duplication, and were subjected to frequent sequence exchange. Transformation of the leaf stripe susceptible cv. Golden Promise with two Rdg2a-candidates under the control of their native 5′ regulatory sequences identified a member of the CC-NB-LRR gene family that conferred resistance against the Dg2 leaf stripe isolate, against which the Rdg2a-mediated leaf stripe resistance involves autofluorescing cells and prevents pathogen colonization in the embryos without any detectable hypersensitive cell death response, supporting a cell wall reinforcement-based resistance mechanism.

Conclusions: This work reports about the cloning of a resistance gene effective against a seed borne disease. We observed that Rdg2a was subjected to diversifying selection which is consistent with a model in which the R gene co-evolves with a pathogen effector(s) gene. We propose that inducible responses giving rise to physical and chemical barriers to infection in the cell walls and intercellular spaces of the barley embryo tissues represent mechanisms by which the CC-NB-LRR-encoding Rdg2a gene mediates resistance to leaf stripe in the absence of hypersensitive cell death.

Introduction

Leaf stripe disease on barley (H. vulgare) is caused by the seed-transmitted hemi-biotrophic fungus Pyrenophora graminea (anamorph Drechslera graminea) [(Rabenh. ex. Schlech.) Shoemaker]. The disease causes severe yield reductions at high infection rates, especially in organic farming systems [1,2]. The fungal mycelia survive in seeds between the parenchymatous cells of the pericarp, and in the hull and the seed coat, but not in the embryo [3]. During seed germination, the hyphae begin to grow intercellularly within the coleorhiza, and then into the embryo structures, the roots and scutellar node, to establish infection in the seedling. During this first colonization phase the pathogen behaves as a biotroph and degrades host cell walls using hydrolytic enzymes without causing cellular necrosis [3–5]. Once infection spreads into the young leaves, growth switches to a necrotrophic phase with the production of a host-specific glycosyl toxin [6] that causes longitudinal dark brown necrotic stripes between the leaf veins, as well as spike sterility. Spores produced on the infected leaves of susceptible plants spread to infect nearby plant spikes.

Race-specific resistance to leaf stripe is controlled by two known Rdg (Resistance to Drechslera graminea) genes. These genes cause hyphal degeneration in the basal part of the coleorhiza and prevent stripe symptoms from appearing on leaves of young or old plants [3,7,5]. H. spontaneum-derived Rdg1a has been mapped to the long arm of chromosome 2H [8,9] while Rdg2a, identified in H.
vulgaris, has been mapped on the short arm of chromosome 7HS [10]. Both resistance genes have been extensively used in classical breeding, but neither has been cloned. Histological characterization of the Rdg2a-dependent resistance response by [5] showed the termination of P. graminea growth at the scutellar node and basal region of provascular tissue of the barley embryo. The immune response was associated with cell wall reinforcement through accumulation of phenolic compounds and enhanced transcription of genes involved in reactive oxygen species (ROS) production and detoxification/protection, but no localized programmed cell death (PCD), which is typically seen in race-specific immune responses [11], was apparent.

In this study we describe the cloning of Rdg2a and the molecular characterization of the Rdg2 locus. Bacterial artificial chromosome (BAC) and cosmid libraries respectively derived from barley cvs. Morex (which is susceptible to leaf stripe) and Thibaut (the donor of the Rdg2a allele) were used for physical mapping of the locus, leading to the identification of three Rdg2a candidates representing sequence-related members of a gene family. Transformation experiments showed that a coiled-coil, nucleotide-binding site, leucine-rich repeat (CC-NB-LRR) encoding gene confers Rdg2a-specific resistance. Similar to that of other R proteins [12], the RDG2A protein localized to the nucleus and the cytoplasm, while histological analysis confirmed that RDG2A involves cell wall-localized autofluorescence and does not trigger a hypersensitive cell death, consistent with physical/chemical defences mounted by the living cells stopping the intercellularly growing leaf stripe pathogen.

Results

Genetic and physical map of the Rdg2a locus

The Rdg2a locus resides in a chromosome region of high recombination [7], which is a characteristic that would assist in map-based cloning. To investigate the molecular basis of the Rdg2a-based P. graminea resistance in barley, map-based isolation of Rdg2a was initiated by constructing a high resolution genetic map representing 2,800 F1 gametes. The locus was delimited to a 0.14 cM marker interval, and a PCR-based marker located 0.07 cM from Rdg2a was developed [7].

Leaf stripe isolate Dg2, which is recognised by Rdg2a [10] (Table S1), is virulent on cv. Morex, indicating that this cultivar does not contain a functional Rdg2a allele. However, due to the availability of a Morex BAC library [13], we took advantage of this resource for marker development. Utilization of the Morex BAC library for marker development and recessive allele isolation is an approach that was previously used for the isolation of homologues and functional alleles at the Mla powdery mildew resistance locus in barley [14–16]. Screening of the library with a probe derived from the CAPS marker MWG851 (Methods S1), allowed identification of BAC clones 146G20, 244G14 and 608H20 that were subjected to end sequencing (Methods S1). The 146G20 and 608H20 clones were also subjected to low-pass (0.3-fold) shotgun sequencing and nine additional CAPS, dCAPS or RFLP markers were identified (Figure 1A; Table S2). Two of these (146.60-1-2 and 146.9-3-6) showed complete linkage with Rdg2a. These PCR-based markers were tested on the three BAC clones, allowing the markers to be located to sections of the contig (Figure 1B). The estimated size of the 146G20 insert was about 140 kbp. 146.1F-1R and 146.4F-3R markers mapped 0.32 cM apart (9 recombinants out of 2,800 gametes), indicating a genetic to physical ratio of about 440 kb per cM in this Rdg2a interval.

To clone the region containing the Rdg2a resistance gene, we constructed a genomic cosmid library of the Rdg2a-containing cv. Thibaut (Methods S1). Screens using markers 146.9-3-6 and 608.32-3-4 identified the clones 95-3-3 and 17-1-1. Analysis of these two clones with other PCR markers from the region indicated that the clones spanned the Rdg2a interval bounded by the closest flanking genetic markers (Figure 1C). The two cosmids which overlapped by 5.9 kb were sequenced, providing a contiguous sequence of 72,630 bp. In BLASTX analyses, the sequenced region was shown to contain three gene models with similarity to plant R genes encoding NB-LRR proteins (GenBank accession number HM124452). The three NB-LRR encoding

![Figure 1. Genetic and physical maps of the Rdg2a locus.](image-url)

(A) Genetic map of Rdg2a. Crossovers identified in the 1,400 F2 plants from a cross between Thibaut (Rdg2a) and Mirco [7] are shown at the top (CO). Orientation is indicated by Tel (telomere) and Cen (centromere). (B) Contig of Morex BAC clones. (C) Thibaut cosmid contig and genes at the Rdg2a locus. Transcription direction of the genes are indicated by arrows.

doi:10.1371/journal.pone.0012599.g001
genes were predicted using the AutoPredgeneset tool of the RiceGAAS software (http://ricegaas.dna.affrc.go.jp/, [17]) and designated Nbs1-Rdg2a, Nbs2-Rdg2a and Nbs3-Rdg2a with their relative locations shown in Figure 1C.

RFLP analysis of BamHI-digested genomic DNA with probes derived from the NB-LRR genes detected only one fragment of about 50 kbp in the resistant cv. Thibaut and in NIL376 containing Rdg2a (Figure S1), which agreed with the 52 kbp fragment size predicted from the sequence assembly (Figure 1C).

In susceptible genotypes, either three fragments were detected (cv. Mirco and Golden Promise) or a single ~20 kbp fragment was detected (cv. Morex) indicating large deletion(s) in this last genotype.

**Structure of Rdg2a candidate genes**

All three Rdg2a candidates were found to be transcribed in resistant embryos, and the transcript structures (Figure 2A) were determined by random amplification of cDNA ends (RACE) and RT-PCR. Nbs1-Rdg2a and Nbs2-Rdg2a had single introns of 217 or 205 bp in the 5' UTR, and predicted full-length NB-LRR protein products of 1,232 and 1,158 amino acids, respectively. The Nbs3-Rdg2a transcript contained a repeat structure, comprising similarity to a full-length NB-LRR protein followed by similarity to part of a NB domain and a full LRR domain (Figure S2). However, the following observations lead us to conclude that Nbs3-Rdg2a encodes only predicted truncated proteins. In addition to a 305 bp intron in the 5' UTR and a 70 bp intron in the 3' UTR, Nbs3-Rdg2a had one 44 bp intron located shortly after the start codon, which was spliced out in only a third (4/12) of the RACE clones analysed. Splicing of the intron causes a frame-shift, resulting in termination after the first 37 amino acids and addition of one novel amino acid (Cys), while retention of this intron results in termination after the first four and a half LRR units (725 amino acids) due to a nonsense substitution mutation (Figure 2A). We thought it unlikely that Nbs3-Rdg2a encodes a functional resistance protein so we did not pursue it further as an Rdg2a candidate.

Apart from the major structural differences, the ORFs of the three genes were 87–90% identical to one another at the DNA level and 81–86% identical and 91–93% similar at the protein level. Comparisons of the 5' untranslated regions showed that Nbs2-Rdg2a and Nbs3-Rdg2a were 93% identical in the 1,040 bp preceding the transcription start point (Figure S2), apart from a 347 bp insertion in Nbs2-Rdg2a, 145 bp upstream of the transcription start site. These findings suggest that the Rdg2a locus arose by gene duplication. A BLASTn search of the Triticeae Repeat Sequence (TREP) database (http://wheat.pw.usda.gov/ITM1/Repeats/) revealed 88% sequence identity between the insertion in the predicted promoter region of Nbs2-Rdg2a and members of the Stowaway class of miniature inverted transposable elements (MITEs). In contrast, Nbs1-Rdg2a showed only weak identity (51%) to the other two genes in the 700 bp preceding the transcription start (Figure S2). The three genes showed no significant similarity in the 3'-untranslated regions.

To provide a comparison with a susceptible (rdg2a) genotype, we used gene-specific primers designed on the Thibaut Nbs1-Rdg2a and Nbs2-Rdg2a genes to obtain genomic sequences from cv. Mirco (GenBank accession numbers HM124453 and HM124454, respectively). Primers based on Nbs1-Rdg2a and Nbs2-Rdg2a genes yielded Mirco sequences with affiliation to the corresponding genes in Thibaut (Figure S2), suggesting that the amplified genes represented true alleles of the Thibaut genes. PCR markers based on insertion/deletions identified in the putative regulatory regions of the two genotypes (see below; Table S2), co-segregated with the Rdg2a locus in the high resolution mapping population (Figure S3, Methods S1), confirming that these two Mirco genes derive from the rdg2a locus.

Neither Mirco gene appears to be transcribed (see below), and this inactivity may be due to structural differences in the 5' sequences (Figure 2B). Mirco Nbs1-rdg2a has a 436 bp insertion next to a putative TATA-box element, and a 854 bp insertion in the 5' UTR with terminal inverted direct repeats of 138 bp. Neither insertion showed similarity to a known transposable element. Mirco Nbs2-rdg2a contained a 41 bp direct repeat just upstream of the transcription start site and lacked the MITE element present in the Thibaut gene (Figure 2B). Mirco Nbs1-rdg2a also contains frame shift mutations, resulting in a severely truncated ORF, whereas Mirco Nbs2-rdg2a contains an intact CC-NB-LRR ORF (Figure S4).

*Nbs2-Rdg2a expression, but not Nbs1-Rdg2a, is pathogen responsive*

Semi-quantitative RT-PCR was performed using primer combinations specific for the Nbs1-Rdg2a and Nbs2-Rdg2a genes in either cv. Mirco or NIL376-Rdg2a (Figure 2C; Table S3). In the susceptible cv. Mirco, neither gene showed detectable expression in embryos or leaves, even after increasing the number of PCR cycles and trying other primer combinations. In NIL376-Rdg2a, expression of both genes was observed in uninoculated control embryos and in leaves of pathogen free plants. Some increase in transcript levels by 7 days after inoculation was evident for Nbs2-Rdg2a but not for Nbs1-Rdg2a (Figure 2C). Therefore, we performed quantitative RT-PCR in embryos of NIL376-Rdg2a at five time points (7, 14, 18, 22 and 28 dai) (Fig. 2D). Nbs2-Rdg2a expression was significantly increased by inoculation at 7, 14, 18 dai (P<0.05, Methods S1) and was unresponsive by 22 dai, while Nbs1-Rdg2a expression was not appreciably altered by leaf stripe inoculation (Figure 2D).

**Identification of Rdg2a**

Genomic clones of the two Rdg2a candidates containing their native 5' and 3' regulatory sequences were used to transform the leaf stripe susceptible barley cv. Golden Promise. Ten randomly chosen T1 lines for each transgene were allowed to self-pollinate and the resulting T1 plants tested for resistance to isolates Dg2 and Dg5. This revealed that lines bearing the Nbs1-Rdg2a transgene were resistant to leaf stripe isolate Dg2 (Table 1). The overall escape rate of 5% among the null segregants was similar to the value observed in the susceptible control varieties (data not shown). Within T1 families, resistance to the same isolate co-segregated with the Nbs1-Rdg2a transgene and its expression (Figure 3A). These lines were susceptible to leaf stripe isolate Dg5, which is not recognised by Rdg2a (Table 1). T1 lines containing the Nbs2-Rdg2a transgene were fully susceptible to both the leaf stripe isolates (Table 1), although RT-PCR confirmed the transgene was expressed (data not shown).

Rdg2a resistance terminates fungal growth in the embryo [5]. In the line 16/S1-T6 containing the Nbs1-Rdg2a transgene, plants challenged with the *P. graminea* isolate Dg2 showed no leaf stripe symptoms and there was no fungal mycelium in the leaves, indicated by undetectable transcripts of two fungal genes coding for *Ubiquitin* and *GTPase activator* (Figure 3B). In contrast, leaf stripe symptoms and fungal transcripts were observed in leaves of 16/S1-T6-rdg2a plants infected with Dg2 or Dg5 and 16/S1-T6-Rdg2a plants infected with Dg3 (Figure 3B).

As the Nbs1-Rdg2a gene could confer the same resistance specificity as Rdg2a in transgenic plants, we concluded that *Nbs1-Rdg2a is Rdg2a*. 
RDG2A protein structure

The predicted Rdg2a product of 1232 amino acids has an estimated molecular weight of 139.73 kDa. It contains all the conserved NB domain motifs of NB-LRR proteins defined by [18,19], including the P-loop, RNBS-A, Kinase 2, RNBS-C, GLPL, RNBS-D and MHD domains, the latter of which is duplicated (Figure 4). A COILS analysis indicated the presence of a potential coiled-coil (CC) domain between amino acids 25 and 60, indicating that RDG2A belongs to the CC subset of NB-LRR resistance proteins [18]. The LRR region contains 22 imperfect repeats with a few repeats showing good agreement with the consensus motif LxxLxLxx(C/N/T)xxLxxLxxLP for cytoplasmic LRRs (Figure 4) [20].

Table 1. Analysis of transgenic plants.

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<th>No. res. plants</th>
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*Made by transforming the susceptible barley cv. Golden Promise with the Rdg2a candidates Nbs1-Rdg2a or Nbs2-Rdg2a. Only those plants containing a transgene copy are included; null segregants are excluded.

*Number of transgenic T1 plants without leaf stripe symptoms. Data were pooled from three independent experiments each comprising 5 or more plants per line.

*Total number of plants tested as controls.

doi:10.1371/journal.pone.0012599.g002

doi:10.1371/journal.pone.0012599.t001
proteins (accessions BAD08990, EEE69085, EEC83970, BAD0894, and BAF24312; Figure 5) encoded by genes clustered in a 2.97 Mbp region of rice chromosome 8 (nt. 25,872,241 to 28,845,527 of AP008214), which is not co-linear with the barley Rdg2a interval [7]. Of the known resistance proteins from barley, low levels (around 16%) of identity, restricted to the conserved motifs of the NB domain, were observed with the MLA1, MLA6 and MLA12 powdery mildew resistance proteins (Figure 5).

Figure 3. Analysis of T1 family 16/S1-T6 segregating for the Nbs1-Rdg2a transgene. (A) T1 seeds were inoculated with P. graminea isolate Dg2 and plants analyzed for disease symptoms in leaves (upper panel), an STS marker for Rdg2a (middle panel; upper band represents the rdg2a susceptibility allele from cv. Golden Promise while the lower band represents the Rdg2a transgene or endogenous gene), and Rdg2a transgene or endogenous gene expression by RT-PCR (lower panel). Resistance (R) or susceptibility (S) status of the plants is indicated underneath. The resistant cv. Thibaut and the susceptible cv. Golden Promise provide controls. (B) Leaves of six 16/S1-T6 T1 plants were analyzed for expression of the fungal (Pg) Ubiquitin and GTPase activator genes and the barley (Hv) Rdg2a gene by RT-PCR. Seeds had been inoculated with Dg2 or Dg5 leaf stripe isolates or were non-inoculated (C). The barley β-actin gene was used as an internal control. Plant DNA was also tested for the presence of the transgene using the Rdg2a STS marker described in (A). doi:10.1371/journal.pone.0012599.g003
The RDG2A and NB2-RDG2A proteins are 75.3% identical, and differences include a deletion of three consecutive LRRs in NB2-RDG2A (Figure S5). Similarity is higher in the CC region than in the NB or LRR regions (Figure 4; 92.6 versus 73–74%), and the proportion of non-conservative amino acid substitutions is lower in the NB domain (75/104 = 72%) than in the LRR domain (57/71 = 80%). Similarly, the ratio of non-synonymous ($K_a$) to synonymous ($K_s$) nucleotide substitutions between Rdg2a, Nbs2-Rdg2a and Nbs3-Rdg2a (longest ORF) is 0.99, 2.13 and 2.63 for the CC, NB and LRR regions, respectively. Within the LRR domain, non-conservative substitutions are about twice as frequent in the b-strand/b-turn xxLxLxx motifs (solvent-exposed residues framed by aliphatic residues [20]) (Boxed, Figure S5) than elsewhere (25/133 = 18.8% versus 32/373 = 8.5%). These comparisons indicate that Rdg2a and its paralogues have been subjected to the highest level of diversifying selection in the LRR-coding region, consistent with the LRR domain being an important determinant of resistance specificity [21].

Localization of RDG2A and NB2-RDG2A proteins to the nucleus and cytoplasm

RDG2A does not have any predicted transmembrane domain or signal peptide sequence, suggesting a cytoplasmic location of the protein. To determine the subcellular location of the RDG2A and NB2-RDG2A proteins, we made 3' fusions with the Yellow Fluorescent Protein (YFP) ORF and expressed the chimeric genes behind the maize polyubiquitin promoter. When either construct...
was transiently expressed in leaf epidermal cells of barley cv. Golden Promise, YFP fluorescence was clearly observed throughout the nucleus and also in the cytoplasmic strands (Figure 6). YFP alone has no nuclear localization signal but is smaller than the 40–60 kDa size exclusion limit of the nuclear pore complex [22]. Consistent with these characteristics, YFP expressed by itself was abundant in the cytoplasm and was also present in the nucleus (Figure 6).

Rdg2a resistance does not involve hypersensitive cell death

Rdg2a-mediated resistance terminates fungal growth coincident with the appearance of cell wall-associated host-cell autofluorescence in tissues containing hyphae, mainly at the junction of the scutellum and scutellar node of the inoculated embryos [5]. Whole-cell autofluorescence is regarded as an indicator of HR in race-specific resistance of barley leaf epidermal cells to powdery

![Image of phylogenetic tree](image-url)

**Figure 5.** Neighbor-joining phylogenetic tree including RDG2A and similar resistance proteins and resistance gene analog products. Numbers on the branches indicate bootstrap percentages. Prefixes indicate species origin. The *A. thaliana* RPM1 protein (Q39214) was used as an outgroup. Shown are the rice (*Oryza sativa*) disease resistance-like proteins BAF24312, BAD08984, BAD08990, EEC83970 and EEE69085, the PM3 wheat powdery mildew resistance protein, products of the *S. bulbocastaneum* blight resistance gene Rpi-blb1 and its paralogues Rga3-blb, and Rpi-blb1, predicted products of RGA_B149.blb, RGA_T118-tar (*S. tarijense*), RGA_SH10-tub (*S. tuberosum*) and Rpi-pta1 (*S. papita*), the I2 and I2C-1 proteins encoded by the tomato (*Lycopersicon esculentum*) I2 resistance locus to Fusarium wilt, the soybean (*Glycine max*) *Phytophthora* root rot resistance protein RPS-L-K-1, and the barley (*H. vulgare*) powdery mildew resistance proteins MLA1, MLA6 and MLA12.

doi:10.1371/journal.pone.0012599.g005

![Image of sub-cellular localization](image-url)

**Figure 6.** Sub-cellular localization of RDG2A and NB2-RDG2A proteins. Barley cv. Golden Promise epidermal cells were transiently transformed with constructs expressing RDG2A:YFP and NB2-RDG2A:YFP fusion proteins (A and D respectively), driven by the maize polyubiquitin gene promoter. A construct expressing YFP alone with the same promoter was used as control (G). Fluorescence signals were visualized using confocal laser scanning microscopy (A, D and G). Bright field images (B, E and H) and merged images (C, F and I) are shown. Scale bar represent 50 μm.

doi:10.1371/journal.pone.0012599.g006
mildew [23,24] but was only occasionally (one or two cells per embryo section) observed in barley embryos expressing Rdg2a resistance. Nuclear DNA fragmentation is another PCD marker in plants [25]. However, while electrophoretic analysis of embryo DNA failed to detect it in association with Rdg2a resistance (data not shown), it is possible that DNA laddering went undetected due to the small proportion of pathogen-challenged cells that would have been present in the sample (cf. Figure 7). Therefore, we further tested for the presence of individual cells undergoing programmed death in the Rdg2a resistance response in situ, by using terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL). This method enables detection of free 3’-OH groups created by DNA strand breaks that occur with programmed cell death. TUNEL was performed on serial sections of NIL3876-Rdg2a barley embryos (Figure 7). In non-inoculated embryos, no autofluorescence was observed under UV light (Figure 7A to C). In inoculated embryos, UV-autofluorescent tissues were observed at the scutellar node and provascular tissue at 14, 22 and 26 dai (Figure 7G, H and I respectively). Calcofluor staining and bright field observations revealed the presence of fungal mycelium in the tissues immediately adjacent to the autofluorescent regions (Figure 7S and T, respectively), indicating that autofluorescence was a genuine defence-associated response against leaf stripe. TUNEL revealed some nuclear DNA fragmentation (bright green fluorescent nuclei) in the coleoptile and in a few cells at the scutellar node of both non-inoculated (Figure 7D to F) and inoculated embryos (Figure 7J to L and M to O), however inoculation had no detectable effect on the frequency of these TUNEL signals. In the scutellar node and basal region of provascular tissue of the inoculated sample we observed, on average, 500 cells per section and time point of inoculation that were in contact with the fungus (on the basis of the calcofluor staining and bright field observations) and only one to two nuclei were positive to TUNEL staining. The same frequency of TUNEL positive nuclei was detected in the same regions of non inoculated embryos. Staining of the same sections with 4,6-Diamidino-2-phenylindole (DAPI) dihydrochloride, verified the presence of intact nuclei in the autofluorescent regions (Figure S6). Following treatment of sections of control or inoculated embryos with DNaseI, TUNEL analysis stained all nuclei (Figure 7P to R), and

Figure 7. Histological analyses of NIL3876-Rdg2a barley embryos. (A) to (C) Sections of embryos grown under control conditions observed under UV excitation. (D) to (F) Sections in (A) to (C) subjected to TUNEL analysis. (G) to (I) Sections of embryos inoculated with leaf stripe isolate Dg2 and observed under UV excitation. (J) to (L) Sections in (G) to (I) subjected to TUNEL analysis; the bright green fluorescence at the level of scutellar node and provascular tissue is due to cell wall autofluorescence. (M) to (O) Magnified views of the boxed regions in (J) to (L) and (G) to (I), (S) and (T) Magnified views of the smaller box in (I) stained with calcofluor (S) or observed under bright field (T); arrows indicate the intercellularly growing P. graminina mycelium. (U) and (V) Magnified views of the small box in (C) stained with calcofluor (U) or observed under bright field (V). (P) and (Q) Respectively, sections of control and inoculated embryos at 26 dai, treated with DNase I and subjected to TUNEL analysis. (R) A magnified view of the region boxed in (Q). White arrows in Figure 7E, K and R indicate TUNEL positive nuclei. Scale bars represent 200 μM (A) to (L), 50 μM (M) to (O) and 25 μM (S) to (T). co = coleoptile, pt = provascular tissue, sa = shoot apex, sn = scutellar node.

doi:10.1371/journal.pone.0012599.g007
no positive signals were observed in sections not treated with the deoxynucleotidyltransferase enzyme (data not shown), indicating that the TUNEL assay was working effectively.

**Discussion**

**Evolution of the Rdg2a resistance locus**

Rdg2a resides in a gene cluster, as does many other resistance genes. This organization can promote unequal recombination, which results in sequence exchange between paralogs and generation of recombinant genes with new resistance gene specificities, as well as expansion/contraction of gene copy number [26]. At the Rdg2a locus, paralogs appear to be the result of relatively recent gene duplication as indicated by the strong DNA sequence identity between the three NB-LRR genes that, in the case of Nbs2-Rdg2a and Nbs3-Rdg2a, extends into the 5' untranscribed region (Figure S2). The unusual structure of Nbs3-Rdg2a, in which sequences encoding part of the NB and the LRR regions are duplicated, together with the deletion of the region containing three complete LRR units in NB2-RDG2A relative to RDG2A, provide further examples of variation at Rdg2 locus generated by recombination.

Diversifying selection also contributes to sequence diversity at R gene loci [27]. However, this may only be the case for R genes that encode receptors that directly interact with pathogen effectors. R genes encoding proteins that act via an indirect guard mechanism, like RPM1 in Arabidopsis, are under conservative rather than divergent selection [28–30]. The functional alleles of these R genes would be conserved through evolution because they detect the presence of avirulence gene products that may not be able to mutate without a fitness penalty to the pathogen [31,32]. Conversely, genes subjected to strong diversifying selection, like wheat Pm3 or barley Mla alleles for race-specific powdery mildew resistance [33,34], and Arabidopsis RPP13 alleles for downy mildew resistance [35] in which sequence diversity is accompanied by functional diversity in pathogen recognition, are speculated to act through a model of direct interaction between R gene and Avr gene products [31,36]. Our finding that Rdg2a was subjected to diversifying selection is consistent with a model in which the R gene co-evolves with a pathogen effector(s) gene, due to direct interaction of the two gene products. In this model, small conformational changes in the RDG2A protein restore the interaction with variant versions of the avirulence gene product, during an arms race between plant and pathogen. In such a model, genes for the leaf stripe avirulence products detected by RDG2A would also be under diversifying selection, similar to avirulence genes characterized in flax rust [37] and Arabidopsis downy mildew [38]. This view is also supported from the observation that in the only two leaf stripe susceptible barley genotypes analyzed to date, Mirco and Morex, sequences highly homologous to Rdg2a are present in syntenic position. In the barley cv. Morex, sequences sharing more than 93% of identity to Rdg2a were identified both in coding and non-coding regions and deletion(s) of intergenic regions and of members of the gene family (data not shown) are responsible of the rearrangements suggested by the Southern analysis (Figure S1).

Despite the fact that Nbs2-Rdg2a contains a complete open reading frame and is expressed in embryos, transgenic expression of Nbs2-Rdg2a failed to confer resistance to leaf stripe isolate Dg2. Analysis of near-isogenic lines indicated that the Rdg2a locus controls partial to strong resistance to at least 4 other isolates of the leaf stripe pathogen (Table S1). Whether the Nbs2-Rdg2a gene contributes any of these other resistance specificities is under investigation using the transgenic lines. The NB2-RDG2A and RDG2A proteins had multiple substitution differences in the NB and CC regions. However, there was only one (conservative) amino acid difference in the CC motif, and there were no differences in any of the motifs recognised as being conserved across the CC-NB-LRR class of resistance proteins (not shown).

The LRR domains also showed a number of differences, including the deletion of three LRR units in NB2-RDG2A relative to RDG2A (Figure S5). Variation between R gene alleles or paralogues reported to abolish function include both single amino acid substitutions [39,40] and the absence or substitution of a section of the LRR domain encompassing one to several repeat units [41,42]. Therefore, the substitutions or deletion within the LRR domain of NB2-RDG2A seem like plausible reasons for the absence of a resistance function for this protein. Transcript of Nbs2-Rdg2a was found to be 2 to 16 times less abundant than that of Rdg2a, depending on the time point and inoculation treatment (P<0.05, data not shown). Considering that transcript abundance correlates with resistance activity for the potato NB-LRR late blight resistance gene RB [43] and the rice receptor kinase-like bacterial blight resistance gene Xa5 [44], lower expression of Nbs2-Rdg2a may contribute to its inactivity. This possibility will be explored by testing transgenic plants over-expressing Nbs2-Rdg2a. Complementation was not attempted using Nbs3-Rdg2a, which produces severely truncated proteins, and while a role of this gene in resistance would seem unlikely, we cannot yet rule it out. Insights into the functional consequences of this gene structure may be revealed by a current re-sequencing study, which aims to survey the Rdg2a locus haplotype variability and gene structure in other barley genotypes known to carry Rdg2a resistance specificities.

Strikingly, neither Nbs1-rdg2a nor Nbs2-rdg2a are transcribed in the susceptible cv. Mirco. Given the fitness cost of expressing some R genes [45], unnecessary R genes may become rapidly inactivated [46]. Rearrangements in the promoter region caused by insertion/deletion of transposable elements (Figure 2B) may explain the lack of expression of the Mirco genes. The alleles of Nbs2-Rdg2a are quite similar (93.1% identical), apart from the MITE insertion in the Thibaut allele. The PromH program for the prediction of plant promoters [http://www.softberry.ru/berry.phtml?group = programs&subgroup = promoter&topic = tsp, [47]] identified potential transcription factor binding sites, a TATA box, and a likely promoter within the MITE sequence (data not shown). It is therefore possible that sequences present in the MITE element contributed to the functionalization of this paralog, similar to the transcriptional activation of the rice blast resistance gene Pit by insertion of a Renovator retrotransposon into its 5' region [48]. Although expression of NB-LRR R genes has only seldom found to be responsive to pathogen infection [49,50], transcription of Nbs2-Rdg2a was enhanced up to three fold by 14 days after inoculation by P. graminea-Dg2 (Figure 2D), a time point when several defence-related genes are transcriptionally up-regulated in the Rdg2a genotype [5]. It would be of interest to identify the regulatory sequences of Nbs2-Rdg2a involved in this pathogen responsiveness and determine whether these are located in the MITE insertion.

While the Rdg2a resistance allele from cv. Thibaut is used in breeding and still provides useful field resistance against leaf stripe disease, it is not effective against all isolates (Table S1) [51]. Therefore, identification of further alleles with different resistance specificity should have value, by broadening the range of resistance genes available to breeders and thus delaying the spread of virulent isolates. The cloning of Rdg2a should facilitate this task, by enabling sequencing and expression analysis of homologues from both wild and cultivated barley. Such an approach has led to the identification of functional Pm3 alleles.
from both wild tetraploid and landraces of bread wheat [52,53], allowing a significant expansion of the resistance gene repertoire available against powdery mildew in wheat.

**RDG2A localizes in the nucleus and cytoplasm, and confers resistance in the absence of programmed cell death**

Fluorescence from transiently expressed RDG2A-YFP fusion protein was abundant in the nucleus and was also present in the cytoplasm, suggesting that resistance functions of RDG2A might relate to one or both of these locations. A nuclear activity of a NB-LRR protein mediated by a WRKY transcription factor was previously demonstrated for the powdery mildew resistance protein MLA10 in barley [54]. MLA10 interacts with WRKY1 in the nucleus in the presence of the *Blumeria graminis* effector AVR10, leading to a de-repression of basal defence mechanisms and effective immunity [54]. We previously observed that a WRKY1 allele (designated WRKY38 in [55]), is up-regulated upon *P. graminea*-Dg2 infection [5]. Therefore, it may be worth testing if RDG2A interacts with WRKY38 and whether this interaction is required for the resistance response. It should however be noted that we determined subcellular localization in leaves of uninfected plants, and that the location of the resistance protein might differ in barley embryos inoculated with *P. graminea*. Irrespective of this, the intracellular localization of RDG2A would imply that the recognition of avirulence gene products occurs inside the host cell and that the leaf stripe Avr gene products are transported across the plasma membrane during the infection. This is notable given that the leaf stripe fungus only grows between cells [3,5], suggesting that there must be a mechanism for delivery of the avirulence protein into the host cell. In contrast, several characterized Avr gene products of *Cladosporium fulvum*, a pathogenic fungus of tomato that shares with *P. graminea* cytokinin transport, have been shown to be delivered to the cell cytoplasm [56].

While HR is a common component of resistance gene-mediated defence and often used as surrogate for resistance protein activity, there are a few known cases of NB-LRR genes conferring resistance without HR, at least based on the failure to observe macroscopically visible host cell death. For example, the barley *Mla1* powdery mildew resistance gene can trigger an immune response without macroscopically visible HR [57] although the *Mla12* allele exhibits clearly a necrotic reaction [58]. It has been proposed that the absence of HR associated with resistance to potato virus X governed by the *Rx* gene in potato is because the resistance mechanism is so rapid, preventing accumulation of the avirulence factor to levels that would otherwise trigger a more extensive host response [59]. Similarly, naturally occurring alleles of Arabidopsis *RPS4* or *RPS6* confer bacterial resistance without development of an HR [60]. In the current study, TUNEL positive nuclei were observed in the scutellum and in the coleoptile both in control and inoculated embryos. However, inoculation did not increase the frequency or distribution of these signals. Therefore, these observations most likely reflect cell death that normally occurs with development, as previously observed in barley germinating seeds and in the corresponding cells of the scutellum and coleoptile of maize embryos [61,62]. In HR of barley epidermal cells against the biotrophic powdery mildew fungal pathogen governed by the *Mla12* resistance gene, autofluorescence and accumulation of phenolic compounds is observed throughout the whole host cell [23,24]. Autofluorescence at the junction of the scutellum and scutellar node regions was observed in the resistance response to leaf stripe, but was essentially confined to the cell walls and only occasionally observed throughout a whole cell (this study, [5]). No necrotic tissues or cell collapse was observed under bright views of the embryo regions showing autofluorescence (data not shown), further indicating that hypersensitive cell death did not occur. One could speculate that an HR-associated resistance response would be too damaging to the embryo, and therefore invisible in an evolutionary sense. HR deprives obligate biotrophic pathogens of living host cells required for successful colonization, but may be favourable to the hemibiotrophic leaf stripe pathogen, which obtains nutrients at later stages of colonization by means of hydrolytic degradation of host cell walls. Rdg2a resistance terminates *P. graminis* mycelium growth at the scutellar node and basal regions of provascular tissue of the barley embryos, and is associated with the accumulation of phenolic compounds in the cell walls of the invaded host tissues. These phenolic compounds are the likely source of the cell wall localized autofluorescence. Also, pathogen-induced up-regulation of several genes related to cell wall modification was observed in the resistant NIL but not in the susceptible one [5]. We therefore propose that inducible secretory immune responses, leading to physical and chemical barriers to infection in the cell walls and intercellular spaces of the barley embryo tissues, represent mechanisms by which the CC-NB-LRR-encoding Rdg2a gene mediates resistance to leaf stripe.

**Materials and Methods**

**Plant and fungal materials**

Genetic mapping was performed using 93 F2 recombinants for the 3.47-cM Rdg2a marker interval ABG704-Sc.OP09, previously selected from an F2 population of 1,400 plants made from a cross between barley cvs. Thibaut (resistant, Rdg2a) and Mirco (susceptible, rdg2a) [7]. NIL3876-Rdg2a contains the Rdg2a gene from Thibaut backcrossed into the genetic background of Mirco [10]. Bailey cv. Morex was used for Southern-blot experiments while the susceptible variety Golden Promise was used for transformation tests. The leaf stripe (*P. graminis*) isolates Dg2 (incompatible on Rdg2a) and Dg5 (compatible on Rdg2a) were used in our study. The Dg2 isolate is the most virulent isolate in a previously described collection of monochondial isolates [51]. The *P. graminis* isolates were grown on PDA (Liofilchem, Italy), in Petri dishes at 20°C for 10 days in the dark. Seeds were surface-sterilized in 70% ethanol for 30 s and then in 5% sodium hypochlorite for 15 min prior to inoculation using the ‘sandwich’ technique [63].

**Generation of transgenic barley lines**

Genomic DNA fragments of about 6 kb were used in transformation experiments, and for *Nbs1-Rdg2a* and *Nbs2-Rdg2a*, included 1196 or 985 bp of 5’ untranscribed sequence, and 556 or 638 bp of 3’ untranscribed sequence, respectively. These were PCR amplified using primer sequences provided in Table S4 and Phusion HF Taq DNA polymerase (New England Biolabs), from cosmid 93-9-3 (*Nbs1-Rdg2a*) and cosmid 17-1-1 (*Nbs2-Rdg2a*), subcloned in pDONR201 (Invitrogen) and then transferred to the Gateway (Invitrogen) compatible version of the *Agrobacterium* binary vector pWBVec8 [64]. Inserts were confirmed as having the same sequence as the cosmids clones. Transgenic barley plants were generated by co-cultivation of *Agrobacterium tumefaciens* with immature barley embryos of cv. Golden Promise, as described by [57]. Transgenes were detected by PCR with the gene-specific primer pair Nbs1_25 and Nbs1_26 (Table S3) that amplified a 387 bp fragment in Thibaut and a 500 bp fragment in Golden Promise. Transgene copy number for *Nbs1-Rdg2a* was evaluated by Southern hybridization analysis of genomic DNAs digested with EcoRI and
KpnI, which respectively have one and two restriction sites in the Rdg2a genomic sequence used for transformation. This identified one single copy integration for all the lines but one multiple copy integration for line 8/S1 (data not shown).

Subcellular localization of RDG2A and NB2-RDG2A

To generate the YFP fusion constructs, the coding sequences of Nbs1-Rdg2a and Nbs2-Rdg2a were firstly amplified from the aforementioned pDONR201 entry clones using 15 ng of plasmid DNA with Phusion HF Taq DNA polymerase (New England Biolabs) according to manufacturer’s instructions, and the products transferred into a Gateway destination vector (pUbi-Gateway-cYFP) previously used in barley transient expression studies [65]. The constructs contain the Nbs1-Rdg2a and Nbs2-Rdg2a ORFs 3’-fused with the TFP ORF, behind the maize ubiquitin promoter. Transient gene expression in barley epidermal cells was performed by particle bombardment as previously described by [66]. Fluorescence imaging was performed using a TCS SP2 AOPS confocal laser-scanning microscope (Leica), with the 514-nm Ar/Kr+ ion laser line used to excite YFP, and 525–580 nm used for image collection. Images were collected and processed using the software LCS (Leica). Reference emission spectra of YFP was used to discriminate genuine YFP emission fluorescence from nonspecific background fluorescence.

Histology

Sections of inoculated (14, 22 and 26 dai) and control embryos were fixed in freshly prepared 4% p-formaldehyde in phosphate-buffered saline (PBS) pH = 7 (130 mM NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4) for 12 hours and then stored in 70% ethanol at 4°C until use. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay was performed according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany), and nuclei were stained by incubating in 1 mM 4-Diamidino-2-phenylindole (DAPI) for 20 min. For TUNEL analysis, three independent replicate experiments were performed. Per experiment, six embryos (five sections for each embryo) were observed per time point and inoculation status. For TUNEL assay, a negative control was provided by omitting terminal deoxynucleotidyl transferase enzyme, and a positive control was provided by treating samples with DNase1. For calcifiocor stainings, sections were incubated in 0.01% calcifiocor in PBS pH 7 for 30 min. Samples were observed on an Olympus BX51 microscope with the settings (a) excitation at 451–490 nm and emission at 491–540 for fluorescein, or (b) excitation at 335–380 nm and emission at >420 nm for autofluorescence, DAPI and calcifiocor staining. Images were recorded using an Olympus DP50 microscope digital camera system.

Supporting Information

Table S1 Rdg2a resistance spectrum.
Found at: doi:10.1371/journal.pone.0012599.s001 (0.03 MB DOC)

Table S2 Details of genetic markers.
Found at: doi:10.1371/journal.pone.0012599.s002 (0.04 MB DOC)

Table S3 Sequences of PCR primer sets and annealing temperatures used in the expression analyses.

References


Embryo Immunity to Leaf Stripe