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A CC-NB-LRR gene confers leaf stripe resistance at the *Rdg2a* locus in barley

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Summary

Barley leaf stripe, caused by the seed-transmitted hemi-biotrophic fungus *Pyrenophora graminea*, is a barley disease particularly acute in Nordic countries, during spring sowing, and in the Mediterranean's winter barley districts where causes severe yield losses.

To date, only two *P. graminea* race-specific resistance genes are known: *Rdg1a*, identified in cultivar Vada, and *Rdg2a*, identified in cv. Thibaut. *Rdg2a* confers immunity to at least three different *P. graminea* monoconidian isolates, including the most widespread and virulent Italian isolate *Dg2*, but it is overcome by *Dg5*.

The aim of the present work was to isolate this gene, characterize the *Rdg2a* locus and its evolution and mine the bases of *Rdg2a*-mediated resistance.

In a previous analysis the map-based cloning and the sequencing of the *Rdg2a* locus were carried out. Three homolog *R* genes encoding CC-NB-LRR proteins, that represent the majior class of resistance proteins, were identified at the locus and were named as *Nbs1-Rdg2a*, *Nbs2-Rdg2a* and *Nbs3-Rdg2a* (Bulgarelli *et al.*, 2010). To determine which of the three genes is the *Rdg2a* gene, we started the research investigating their structure. RACE analyses showed that for the third candidate (*Nbs3-Rdg2a*) alternative splicing processes determine the synthesis of severly truncated and probably non-functional proteins. This allowed us to excluded *Nbs3-Rdg2a* from acting in resistance to isolate *Dg2*. By means RT-PCRs on a pair of *N*ear *I*sogenic *L*ines (NIL) that differ only for alleles at the *Rdg2a* locus (susceptible cv. Mirco and its resistant NIL, NIL3876), we demonstrated that both *Nbs1-Rdg2a* and *Nbs2-Rdg2a* are transcribed in embryos (where the resistance takes place) and leaf tissues of the resistant line, but they are not expressed in the susceptible near-isogenic phenotype. Sequencing of the Mirco alleles revealed rearrangements in the putative promoter regions: two insertions, one next to a putative TATA-box element and the other, carrying terminal inverted repeats, in the 5' UTR, for *Nbs1-rdg2a*, and a deletion just at the level of a MITE-like element, present in Thibaut *Nbs2-Rdg2a*, for *Nbs2-rdg2a*. It is likely that these changes represent the cause of the lacking of expression of these genes in the susceptible genotype. Moreover, qRT-PCRs showed that *Nbs1-Rdg2a* transcription was un-responsive to *P. graminea* infection, while

Nbs2-Rdg2a transcripts increased during the first stages of infection. However, *Nbs2-Rdg2a* mRNA abundance was significantly lower than that of *Nbs1-Rdg2a*.

To define which gene is the *Rdg2a* gene, a complementation assay was conducted. Susceptible cv. Golden Promise was transformed by agoinfiltration with each gene independently, under the control of its native promoter. Interestingly, the rescue of *Dg2*-resistance was observed only when plants were transformed with *Nbs1-Rdg2a* (90-100% of resistance), suggesting that this gene is *Rdg2a*.

Comparing the sequences of the three genes at the *Rdg2a* locus, we found that similarly to other *R* genes, *Rdg2a* underwent to diversifying selection, according to a model in which resistance genes co-evolves with pathogen effector(s) gene(s). The fact that the *Rdg2a* locus contains a gene cluster of highly similar sequences has most likely contributed to significative rearrangements during evolution, probably derived from unequal crossovers resulting in sequence exchange between paralogs and, possibly, in the generation of recombinant genes, as well as to expansion/contraction of gene copy number. Regarding this last case, we have also characterized the *rdg2a* locus of the susceptible cv. Morex. Morex *rdg2a* locus carries two deletions and the *rdg2a* allele might derived from an un-equal crossing-over between *Rdg2a* and *Nbs2-Rdg2a* ancestors that led to a reduction of the number of the gene family members.

Most resistance proteins function through inducing a *P*rogrammed *C*ell *D*eath (PCD) that lead to a *H*ypersensitive *R*esponse (HR) at the level of the infected cells. Histological analyses using the TUNEL method did not reveal any significative difference in PCD between infected embryos of resistant and susceptible varieties and the number of cells undergoing PCD was transcurable. These finding let us to conclude that the *Rdg2a*-mediated resistance does not involve HR but it is most likely based on the strengthening of physical and chemical barriers in the cell walls and intercellular spaces of the embryo tissues.

In conclusion, we identified, cloned and characterized the first resistance gene active against a seed-borne disease; importantly, the gene belongs to the poorly represented class of *R* genes which does not trigger a hypersensitive responce.

1. Introduction

1.1 The Plant Immune System

Like animals, plants are engaged in a constant battle against the wide range of microbial pathogens, but conversely to animals, they lack the circulating adaptive immune system and respond to pathogen challenges by developing a cell-autonomous innate immunity system (Rafiqi *et al.*, 2009; Ausbel, 2005). Plant immunity can be divided in two branches: the first is represented by passive defences that include endogenous compounds or barriers such as wall cell; while the second is an active defensive arsenal in turn composed by two branches: the "basal" or "primary" and the "*R*esistance (*R*) gene-mediated" innate immune system (Glowacki *et al.*, 2010; Bent and Mackey, 2007; Chrislom *et al.*, 2006; Jones and Dangl, 2006).

Basal defences are activated by the action of transmembrane *P*attern *R*ecognition *R*eceptors (PRRs), usually *R*eceptor-*L*ike *P*roteins (RLPs) or *R*eceptor-*L*ike *K*inases (RLKs). Most PRRs belong to the LRR-PRR class of receptor carrying a *L*eucine-*R*each *R*epeat (LRR) domain (Par. 1.2.1.1) and are located mainly on the plasma membrane, but also in endosomal compartments or cytoplasm. They resemble animal *T*oll-*L*ike *R*eceptors (TLRs) in terms of their structure and function (Glowacki *et al.*, 2010) and interact with the *M*icrobial- or *P*athogen-*A*ssociated *M*olecular *P*atterns (MAMPs or PAMPs). MAMPs or PAMPs are invariant (surface) structures or molecules indispensable to the microorganism that do not exist in the host which recognizes them as non-self, through the PRRs (Postel and Kemmerling, 2009), and activates MAP kinase signaling cascades leading to the induction of primary defence responses that inhibit colonization of non-adapted, non-pathogenic or non-host pathogens (Panstruga *et al.*, 2009; Bittel and Robatzek, 2007; Jones and Dangl, 2006). This system is referred to as *P*AMP-*T*riggered *I*mmunity (PTI).

The most studied PAMP is the bacterial flagellin which triggers defence responses in various plants. A synthetic 22-amino acid peptide (flg22), from a conserved flagellin domain, is recognized by the LRR-RLK *Fl*agellin *S*ensitive 2 (FLS2), consisting of an N-terminal signal peptide, 28 LRRs, a transmembrane domain, and

a cytoplasmic kinase domain. Upon binding of the ligand, this receptor dimerizes with a related LRR-RLK, BAK1, that positively regulates FLS2 function, and the complex is internalized in a kinase-dependent manner (Postel and Kimmerling, 2009). Thus, a signaling cascade is triggered leading to the activation of many cellular responses, including the rapid transcriptional induction of at least 1100 *Arabidopsis thaliana* genes (Zipfel, 2004). Well characterized PAMPs are also the bacterial N-terminal acetylated 18 and 26 amino acid-long fragments (elf18 and elf26) and *E*longation *F*actor Tu (EF-Tu), recognized by an *Arabidopsis* LRR-RLK called EFR (Tsuda and Katagiri, 2010; Zipfel, 2006). EFR is a homolog of FLS2 and, since these receptors are structurally similar and serve similar functions it is assumed that more members of the LRR-RLK family may be receptors for yet unidentified PAMPs (Postel and Kimmerling, 2009). Other MAMPs triggering PTI in *Arabidopsis* are peptidoglycans (PGNs, components of bacterial cell walls) and chitin (a major component of fungal cell walls) (Boller and Felix, 2009).

Recently, another class of non-LRR receptors attracted common interest: the LysM motif proteins. From bacteria it is known that the LysM domain binds peptidoglycans. A LysM-receptor kinase CERK1/LysM RLK1 was shown to be necessary for perception of chitin oligomers (N-acetyl glucosamine oligomers) that are structurally related to peptidoglycans (N-acetyl glucosamine oligomers/N-acetyl muramic acid backbone with connecting peptide side chains). However, CERK1 chitin-binding activity was not revealed, but it is possible that this protein cooperates with CEBIP for the recognition of chitin. CEBIP is a chitin binding protein identified in rice and consisting of a LysM domains and a transmebrane domain but lacking the kinase signalling domain (Postel and Kimmerling, 2009).

Perception of pathogens must be expanded to the surveillance of the integrity of the plant itself. The so-called *D*anger-*A*ssociated *M*olecular *P*atterns (DAMPs) are signals produced by the host and released upon plant damage. One representative is the *Arabidopsis* AtPep1, a peptide derived from a pro-peptide that is induced after infection and recognized by its cognate receptor PEPR1. By the fact that the RLK Theseus, involved in cell elongation control in *A. thaliana*, seems to control cell integrity and the expression of genes involved in pathogens defense, it was proposed

that alteration of cell integrity might not only be a signal for developmental growth control but also for danger-associated processes (Postel and Kimmerling, 2009).

The second defence takes place once adapted pathogens evolved the ability to suppress the first layer of defence, by delivering on the apoplast and/or in host cells effectors able to interfere with the basal responses (Rafiqi *et al.*, 2009). As examples, in *Arabidopsis*, the *Pseudomonas syringae* AvrPto directly inhibits the intracellular kinase signaling domains of several PRRs (Xiang *et al.*, 2008), and also the downy mildew proteins ATR1 and ATR13 promote disease susceptibility in this specie (Sohn *et al.*, 2007). By this way and with additional effectors, that make use of the host's nutrients, pathogens can survive and complete their life cycle (Block *et al.*, 2008; Alfano and Collmer, 2004). This phenomenon is called *E*ffector-*T*riggered *S*usceptibility (ETS). In the dynamic co-evolution of host-pathogen interaction, plants have acquired highly specific cognate *R*esistance (R) proteins that either directly or indirectly recognize pathogen effector proteins. Thus, virulence factors are turned into *Avi*rulence (Avr) factors that allow the plant to specifically detect formerly successful pathogens (Postel and Kemmerling, 2009). This second mode of immunity is named *E*ffector-*T*riggered *I*mmunity (ETI). ETI triggers a strong disease resistance by activating basal defence reactions, and often the *H*ypersensitive *R*eaction (HR) that implies *P*rogrammed *C*ell *D*eath (PCD) at pathogen infection sites (Jones and Dangl, 2006; Dangl *et al.*, 1996). HR typically does not extend beyond the infected cell: it may retard pathogen growth in some interactions, particularly those involving haustorial parasites, but, for ETI, is not always observed nor required. It is unclear what actually stops pathogen growth in most cases (Jones and Dangl, 2006).

Although engaging different molecular receptors and activating different signaling pathways, PTI and ETI networks are believed to interconnect to stop pathogen infection (Rafiqi *et al.*, 2009; Panstruga *et al.*, 2008; Truman *et al.*, 2006). Jones and Dangl (2006) represented the plant immune system as a four phased “zigzag” model (Fig. 1.1): in phase 1, PAMPs or MAMPs are recognized by PRRs, resulting in PTI that can halt further colonization. In phase 2, successful pathogens deploy effectors that contribute to their virulence. These effectors can interfere with PTI, leading to ETS. In phase 3, a given effector is “specifically recognized” by one or more R

proteins that trigger ETI. Recognition is either direct or indirect. Actually, ETI can be considered as an accelerated and amplified PTI response, resulting in disease resistance and, usually, HR at the infection site. In phase 4, natural selection drives pathogens to avoid ETI either by shedding or diversifying the recognized effector gene, or by acquiring additional effectors that suppress plant defence response. By contrast, plants also ungergo to natural selection developing new R specificities so that ETI can be triggered again.

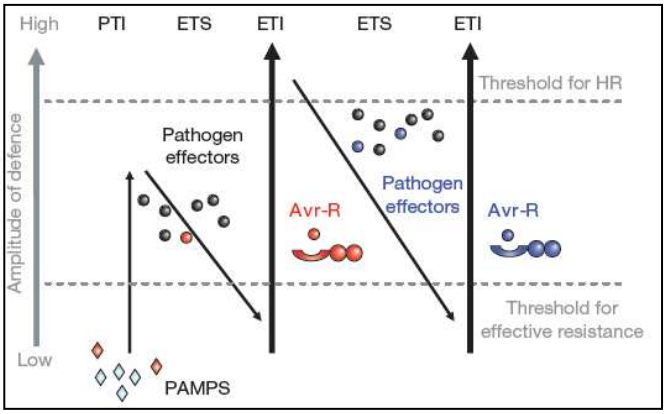


Fig. 1.1 A zigzag model illustrates the quantitative output of the plant immune system. In phase 1 plants detect MAMPs/PAPMPs (red diamonds) through PRRs to activate PTI. In phase 2, successfull pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in ETS. In phase 3, one effector (indicated in red) is recognized by an R protein, activating ETI that often passes a threshold for induction of HR. In phase 4, pathogen isolates are selected that have lost the effector, and perhaps gained new effectors though horizontal gene flow (in blue); these can help pathogens to suppress ETI. Selection favours new plant R alleles that can recognize one of the newly acquired effectors, resulting again in ETI (Jones and Dangl, 2006).

1.2 Resistance (R) proteins

*R*esistance (R) proteins are encoded by the so called *R*esistance (*R*) genes. One class of these genes encodes membrane bound proteins with an extracellular LRR domain (Par. 1.2.1.1), either with or without an intracellular kinase domain. The corresponding Avr proteins are secreted into the apoplastic space during infection, where they may be detected (Rafiqi *et al.*, 2009). However, the majority of known *R* genes encode intracellular proteins with a LRR domain and a *N*ucleotide-*B*inding (NB) domain, connected by a region called ARC (Par. 1.2.1.2; Glowacki *et al.*, 2010). These are among the most numerous proteins found in plants with about 150

Nbs-LRR genes found in *Arabidopsis* and about 600 in rice (Rafiqi *et al.*, 2009; Goff e*t al.*, 2002). The NB-LRR proteins, ranging from about 860 to 1900 amino acids (McHale *et al.*, 2006), belong to a subgroup of the STAND (*S*ignal *T*ransduction *A*TPases with *N*umerous *D*omains) family (Lukaski *et al.*, 2009) and they directly or indirectly recognize specific Avrs (Caplan *et al.*, 2008; Bittel and Robatzek, 2007; Mackey and McFall, 2006) prior to intiating the specific resistance response.

Two subfamilies of NB-LRR proteins can be distinguished on the base of the presence of different domains at the N-terminal: the first is characterized by the *T*IR-*N*B-*L*RR (TNL) proteins containing the TIR domain, originnaly identified as an intracellular part of the *Drosophila* Toll and the human *I*nter*L*eukin 1 (IL1) receptors (Par. 1.2.1.3); non-TIR-NB-LRR proteins belong to the second family and contain other domains. The largest group of these proteins is represented by the CNL (*C*C-*N*B-*L*RR) receptors carrying an N-terminal *C*oiled-*C*oil (CC) domain (Tab. 1.1; Glowacki *et al.*, 2010). Such domains are present in various organisms and have an important role in oligomerization processes like the TIR domain (Par. 1.2.1.3; Chen *et al.*, 2007; Palsson-McDermott and O'Neil, 2007; Oakley and Hollenbeck, 2001). So far, no TNL proteins have been detected in monocotyledonous plants. Although analizying rice genome sequence databases made it possible to identify several genes encoding proteins that contain the TIR domain, they do not seem to be related to NB-LRR proteins (Bai *et al.*, 2002). On the other hand, in dicotyledons, TNLs constitute a strongly diversified group in terms of their structure. Analizying the *A. thaliana* genomes it was possible to detect genes showing TNL proper sequences, but with a structure noticeably different from the typical TIR-NB-LRR domain arrangement. For example, the *Arabidopsis* RRS1-R protein (defined resistance to *Ralstonia solanacearum*) contains a C-terminal WRKY-type domain (Par. 1.2.1.3; Tab. 1.1; Deslandes e*t al.*, 2003). Other identified TNL proteins lack the TIR domain at their amino-terminus (NB(TIR)-LRR), and their classification as TNLs is determined by the NB domain sequences (Radwan *et al.*, 2008; Meyers *et al.*, 1999) or the presence of a C-terminal TIR domain (Glowacki *et al.*, 2010). In the *Populus* genome, sequences encoding proteins which most likely contain a TIR domain at each terminal end were detected (Kohler *et al.*, 2008). "Atypical" proteins were also found for the CNL sub-family. *Arabidopsis*, rice and poplar showed proteins lacking

an LRR domain (CC-NB), or containing two LRR domains, or lacking a CC domain but with a classic NB domain (NB(CC)-LRR) (Tab. 1.1; Kohler *et al.*, 2008; Meyers *et al.*, 2003). In addition, in rice, genes encoding proteins with two NB domains (CC-NB-NB-LRR) are present (Tab. 1.1; Zhou *et al.*, 2004). Apart from CC-NB-LRR proteins, the non-TIR-NB-LRR protein sub-family also comprises other smaller sub-families, including BEAF and DREF proteins that contain a zinc-finger DNA-binding domain, BED-NB-LRR or BED-NB, without the LRR domain, proteins (Tab. 1.1; Zhou e*t al.*, 2004; Meyers *et al.*, 2003). In poplar, a group of the so-called "mixed" proteins showing both the N-terminal TIR and CC domains (TIR-CC-NB-LRR) was identified (Tab. 1.1; Kohler *et al.*, 2008).

Domain structure		Example
TIR-NBS-LRR		
	TIR-NBS-LRR	N receptor L6 receptor
	TIR-NBS-LRR-WRKY	RRS1-R receptor
	$NBS_{(TIR)}$-LRR	2 Arabidopsis*
non-TIR-NBS-LRR		
	CC-NBS-LRR	I-2 RPS5
	$NBS_{(CC)}$-LRR	4 Arabidopsis*
	BED-NBS-LRR	Poptr_1:787192
Mixed		
	TIR-CC-NBS-LRR	2 *Populus**
		

Tab. 1.1 Major classes of plant R proteins (Glowacki *et al.*, 2010).

In plants cells. there are other non-NB-LRR R proteins. Examples are those consisting of kinase domain (Pto) or transmembrane helix domains (Xa13 and MLO). These three proteins respectively take part in innate immunity, fertility and programmed cell death. The function of the others is still unknown (Xiao *et al.*, 2008).

1.2.1 Structure of the NB-LRR proteins

The three-dimensional structures of plant resistance proteins are based on the structures of their animal homologs, but advanced technologies in molecular biology and bioinformatics tools have enabled prediction of the structures and mechanisms of interaction of specific receptors with pathogen effectors. Although the two main domains of plant R proteins, NB and LRR, seem to be the most crucial in pathogen recognition and activation of ETI signal transduction, there are evidences that other domains act together in triggering resistance (Glowacki *et al.*, 2010). For example, several recombinants at the flax *L* locus, conferring resistance to rust, combining TIR and NB domains from different alleles resulted in non-functional resistance genes (Luck *et al.*, 2000).

1.2.1.1 The LRR domain

A functional LRR domain is constituted by at least two tandem repeats of 20-30 amino acids containing the consensus LxxLxLxxLNxL, where L is leucine or another aliphatic amino acid, N is asparagin, threonin, serine or cystein, and x is any amino acid (Fig. 1.2(b); Bella *et al.*, 2008; Stange *et al.*, 2008). The terziary structure of a single LRR domain was predicted for the bovine decorin (12 LRR repeats) and is usually formed by a horseshoe-shaped superhelix, with a backbone of parallel β-strands containing hydrophobic residues and an outer part, usually composed of α-helices, connected with the backbone through β-strands by β-turns (Fig. 1.2(a); Bella *et al.*, 2008; McHale *et al.*, 2006).

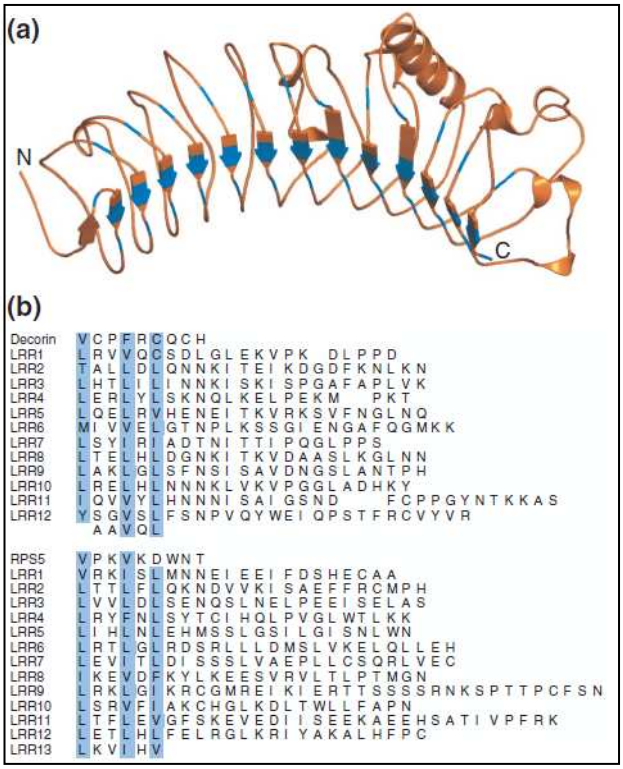


Fig. 1.2 The predicted structure of the LRR domain of the *Arabidopsis* RPS5 onto bovine decorin (PDB code 1xku). **(a)** Cartoon rappresentation of the predicted structure of the LRR domain generated using PyMol. The β-sheets forming the concave face of the "hourseshoe" are represented as arrows. The conserved aliphatic residues are shown in blue. N = amino terminus; C = carboxyl terminus. **(b)** Alignement of the 12 LRRs in decorin and the 13 repeats in RPS5 as well as the amino terminal 9 amino acids. The conserved aliphatic residues are shown in blue (McHale *et al.*, 2006).

Each repeat forms other coils of this superhelix and the "hourseshoe" is the site of specific interaction with other proteins that, in the case of R proteins, provide the determinant for pathogen effector recognition (Glowacki *et al.*, 2010). Notably, differences in the number and the amino acid composition of the repeats determine specificity of this recognition (Bella *et al.*, 2008; Kobe and Kajava, 2000). For example, the variation of LRR copy number in tomato LRR-TM genes *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9*, which confer resistance against *Cladosporium fulvum*, determines their resistance specificity (Liu e*t al.*, 2007). On the other hand, the specific capacity to recognize pathogen of Pi-ta, a CC-NB-LRR protein acting against rice blast, depends on a single amino acid difference in the LRR domain (Jia *et al.*, 2000) and, similarly, six amino acid changes between the flax rust resistance genes *P* and *P2* within the predicted β-strand/β-turn motif of four LRR units establish their resistance specificity (Dodds *et al.*, 2001).

Although the importance of the LRR domain in determing pathogen specificity, it seems it can also function as a regulator of R protein activity (Rafiqi *et al.*, 2009). In particular, it may be an intramolecular inhibitor of the receptor activity when an appropriate elicitor is absent (Glowacki e*t al.*, 2010). It has been proposed that after binding a proper Avr, conformational changes within the LRR domain would take place leading to its dissociation from the NB domain and consequently, to the activation of the receptor (Caplan e*t al.*, 2008; Liu *et al.*, 2007). Neverthless, the dissociation of the LRR and NB domains might not be required for the activation, but repetitive rounds of dissociation and re-association could lead to the amplification of the signal originated from elicitors (Rairdan and Moffett, 2006). In some examples, deletion of the LRR domain in different NB-LRR proteins resulted in autoactivated proteins (Rafiqi *et al.*, 2009). Similarly, a chimeric flax L6 protein carrying a 20-amino acid-long C-terminal fragment from L2 exhibited an autoactive phenotype (Howles *et al.*, 2005). In *RPS5* transgenic tobacco, the LRR and NB domains interact forming an inactive structure (Liu *et al.*, 2007). In contrast, the presence of the LRR domain is required for HR induction of autoactive proteins mutated in the ARC domain, suggesting that the LRR domain can act both as a negative and positive regulator of R proteins in coordination with specific interaction with the ARC domain (Rafiqi *et al.*, 2009).

Altogether, these observations point out that, in the absence of pathogen stimuli, resistance proteins are kept in inactive conformation *via* intramolecular interactions and the presence of the corresponding Avr factor may induce subtle changes in domain interactions rather than a complete dissociation of the different subdomains to trigger activation of ETI (Rafiqi *et al.*, 2009).

1.2.1.2 The NB domain

The NB domain, combined with an ARC motif, is even referred to as *N*ucleotide-*B*inding *S*ite (NBS) or NB-ARC and is present in different proteins involved in cell growth, differentiation, cytoscheletal organization, vescicle transport, apoptosis and defense, such as ATP synthase β subunits, ras protein, ribosomal elongation factors, adenylate kinase, other than R proteins (Liu *et al.*, 2007). To date, the three-dimensional structure of this domain for any plant R proteins has not been

determined; however, it was characterize for the human APAF-1 (*A*poptotic *P*rotease-*A*ctivating *F*actor 1) protein and its *Caenorabditis elegans* homolog CED-4, making it possible to speculate about the structure and the function in plants (Fig. 1.3; Glowacki e*t al.*, 2010). The NB domain of APAF-1 consists of four subdomains: NB containing a P-loop NTPase fold (Lukasik and Takken, 2009), ARC1, ARC2 and ARC3. Instead of ARC3, plant resistant proteins contain a short linker connecting ARC2 with the LRR domain. ARC1 is composed of a bunch of α-helices and binds the LRR domain; while α-helics rolled up in a winged helix fold constitute the ARC2 subdomain (Fig. 1.3; Riedl *et al.*, 2005). Most of the conserved motifs in the NB domain, such as the P-loop, the RNBS-A, kinase 2, RNBS-B, RNBS-C, GLPL, RNBS-D and MHD (McHale *et al.*, 2009; Takken *et al.*, 2006), are present at the interface of the NB, ARC1 and ARC2 domains where they form the nucleotide bindind poket (Fig. 1.3; Lukasik and Takken, 2009). In more detail, the P-loop and the MHD motif bind ADP and orientate it, as well as GLPL motif. The P-loop interacts with ATP and Mg^{2+} ions, as does kinase-2 (Fig. 1.3; McHale *et al.*, 2009; Liu *et al.*, 2007). RNBS-A, RNBS-B and RNBS-D are though to be involved in the hydrolysis of ATP (Tameling e*t al.*, 2006).

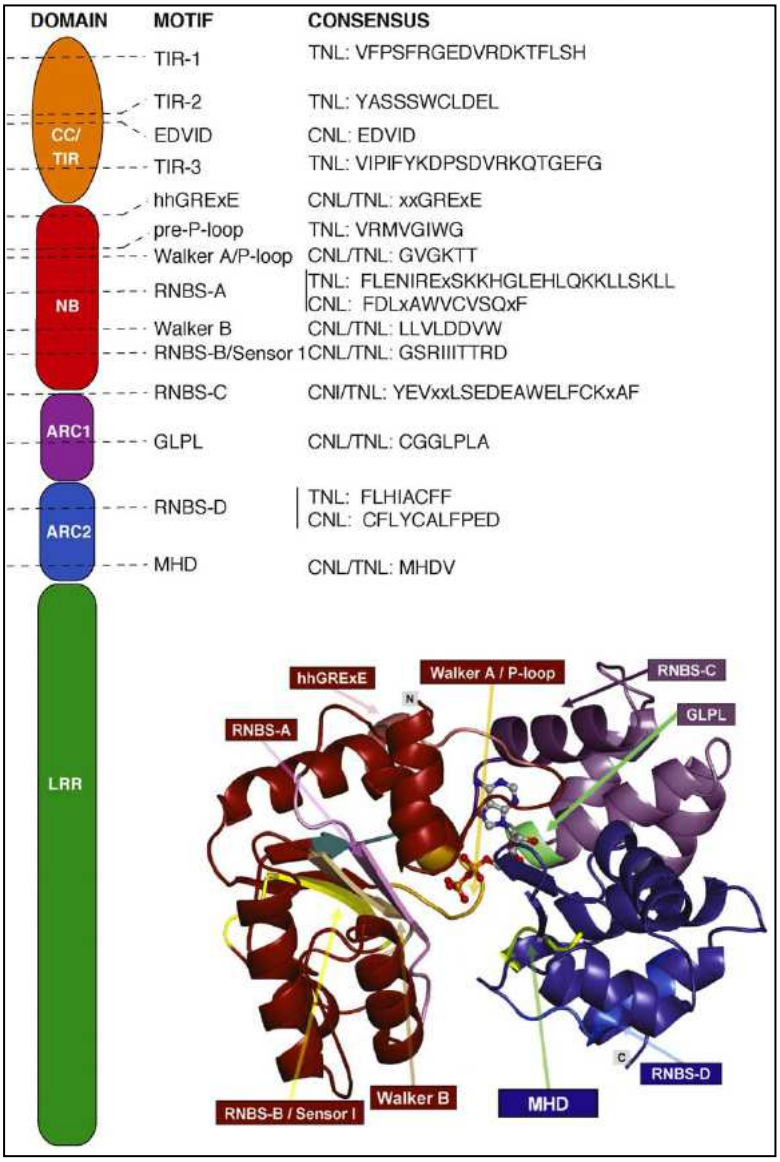


Fig. 1.3 Schematic representation of a typical NB-LRR protein and predicted 3D structure of the NB-ARC domain of tomato NB-LRR I-2 protein modelled on the ADP-bound APAF-1 template. The sub-domains are depicted as coloured boxes, whereas conserved motifs are marked as lines. Consensus sequences are written next to the name of the motifs. In the 3D structure conserved motifs and N- and C-terminal are marked. ADP and Mg atoms are depicted as balls and sticks (Lukasik and Takken, 2009).

The NB domain in TNL proteins differes from the CNL ones for the presence of additional loops (McHale *et al.*, 2009).

In tomato, two CC-NB-LRR proteins, I-2 conferring resistance to *Fusarium oxysporum* and Mi-1 involved in resistance to root-knot nematodes and aphids, were found to bind ATP and the P-loop was essential for this binding (Tameling *et al.*, 2002). Similar results were also observed for the tobacco N protein (Ade *et al.*,

2007) and *Arabidopsis* RPS5 (Bostjan and Andrey, 2001), as amino acid substitution in the P-loop suppressed their activities.

As mentioned in Par. 1.2, proteins carrying an NB-ARC domain belong to the STAND family of NTPases as this domain is characterized by NTPase activity and plays a crucial role as a molecular switch activating signal transduction (Lukasik and Takken, 2009). During the transduction changes in the conformation of the NB domain occur, depending whether it is combined with ATP or ADP, and lead to the activation/deactivation of the whole receptor (Tameling *et al.*, 2006).

The tomato mutants *I-2* and *Mi-2* carry alterations in the NB domain that generate an autoactivation of resistance response in the absence of the elicitor or an increased susceptibility to pathogens. In particular, mutations that suppress the ATP hydrolysis (in the RNBS-A subdomain) constitutively trigger resistance response, demonstrating that the binding with ATP rather than ADP seems necessary to activate the receptor. At the same time, mutations in the region responsible for nucleotide binding bring about a loss in receptor activity (Tameling *et al.*, 2006). Autoactivation was also shown by mutations in the MHD motif of the flax L6 protein, leading to spontaneous necroses (Howles e*t al.*, 2005), and in domain swapping between potato Rx proteins (which recognize the capsid protein of potato virus X) and GPA2 (which recognize the elicitors of pathogenic nematodes) (Moffett e*t al.*, 2002; Rairdan e*t al.*, 2008). On the other hand, loss-of-function mutants defective mainly in the NB and ARC1 subdomains as well as in the ARC2 subdomain were identified (Takken *et al.*, 2006). However, since the low stability of the ARC1 domain, it is possible that the cooperation of some additional factors is necessary for the process (Rairdan *et al.*, 2006). Ueda *et al.* (2006) suggested that the active form of tobacco N protein binds ATP and the ATP hydrolysis is enhanced by the interaction with its elicitor, the tobacco mosaic virus p50 protein. By these observations, it is possible that different R proteins have different modes of activation (Rafiqi *et al.*, 2009).

1.2.1.3 Other domains

Apart from the domains above, as described in Par. 1.2, there are additional domains, usually located at the amino-terminus, and more rarely at the carbossyl-

terminus, in most NB-LRR proteins. The most common are the TIR and the CC domains.

The TIR domain is widely spread among different organisms and was originally characterized due to homology with the intracellular regions of the mammalian IL-1 receptor (IL1-R1) and the *Drosophila* protein Toll that are key mediators of the innate immune responses to bacterial and fungi (Liu *et al.*, 2007). In general, the TIR domain seems to be crucial for the interactions with adaptor molecules mediating the initiation of signal transduction (Janssens and Beyaert, 2003). Interestingly, many factors interacting with animal TLRs also contain a TIR domain, and during these interactions, the TIR domains react with each other physically (Li *et al.*, 2005). Furthermore, TIR domains condition the heterodimerization of some animal TLR receptors (Gautam *et al.*, 2006). An analougous role of this domain has been proposed for plants, even if no adaptors with which it could interact have thusfar been discovered (Glowacki *et al.*, 2010). However, in plants, it is likely to be involved in the recognition of Avr proteins. As proof, the above mentioned binding between tobacco N and p50 proteins accours through the TIR domain of N (Burch-Smith *et al.*, 2007; Mestre and Baulcombe, 2006).

The CC (also called *L*eucine *Z*ipper (LZ)) domain is usually attributed an analogous role to that of TIR domain as a mediator in interactions with other elements of the signaling pathways associated with innate immunity (Glowacki e*t al.*, 2010). It serves as oligomerization domain for a wide variety of proteins including structural and motor proteins and transcriptional factors (Nooren *et al.*, 1999). A research has shown that the conservative motif EDIVD can be identified in the CC domain of all CC-NB-LRR proteins, except for *Arabidopsis* RPS2, RPS5 and Dm3. Mutations in this motif cause disturbances in the intramolecular interaction with the NB and LRR domains, resulting in a decreased resistance response to pathogen attack (Rairdan *et al.*, 2008). Interestingly, the CC domain can bind target proteins for pathogen effectors; *e.g.*, the CC domain of *A. thaliana* RPS5 protein is activated after the interaction with PBS1, which is a target of AvrPhB, the effector of *Pseudomonas syringae* (Ade *et al.*, 2007). Examples of negative regulation do not lack; Belkhadir and co-workers (2004), showed that the CC domain of RPM1 interacts with

Arabidopsis RIN4, a plasma membrane localized and evolutionary conserved protein, to negatively regulate resistance to *P. syringae*.

Given the presence of TIR or CC domain as well as their diversity, the amino-termini of R proteins are though to be involved in protein-protein interactions, possibly with the proteins being guarded or with downstream signaling components (McHale *et al.*, 2006); nevertheless, how the physically interaction occurs remains unclear (Liu *et al.*, 2007).

Plant WRKY transcription factors, identified by the WRKYGQK conservative motif located at the C-terminal of some NB-LRR proteins, along with a typical domain similar to the zinc finger motif, play a crucial role in regulating the expression of genes involved in plant resistance (Eulgem and Somssich, 2007; Ulker and Sommssich, 2004). Some NB-LRR proteins have the ability to affect WRKY transcription factors directly. Alleles of barley MLA proteins recognize the corresponding *Blumeria graminis* Avr, and deactivate HvWRKY1/2 transcription factor, which is a repressor of resistance genes (Liu and Coaker, 2008). As before (Tab. 1.1), there are also NB-LRR proteins containing a domain with the structure of a WRKY transcription factor at their C-terminal, which makes it possible to affect gene expression directly (Glowacki *et al.*, 2010). An example is the RRS-1R receptor of *A. thaliana*, a TIR-NB-LRR protein that recognizes the Pop2 effector of *Ralstonia solanacearum* (Deslandes e*t al.*, 2002). WRKY domain is also present in transcription factors that regulate senescence, trichome development and response to abiotic stresses.

1.2.2 The role of R proteins in plant innate immunity

As mentioned before, the main role for resistance proteins is the activation of signaling transduction that leads to the defence responses, after the recognisement of pathogen effectors. The “gene-for-gene” model defines the direct specific interaction between a pathogen effector and the corresponding host R protein (Fig. 1.4A; Flor, 1971). An example is the flax *L* locus alleles encoding NB-LRR proteins that interact with the AvrL proteins in a two-hybrid assay (Dodds e*t al.*, 2006). Neverthless, in most cases, the cooperation of some host’s additional proteins is necessary to initiate ETI. This phenomenon is explained by the so-called “guard”

model (Fig. 1.4B) which implies that a target protein of the pathogen effector (guardee) is "guarded" by a suitable guard protein, the NB-LRR receptor, and mediates the indirect recognition of the Avr (de Witt, 2007; Tameling and Baulcombe, 2007; Jones and Dangl, 2006). The *Arabidopsis* protein PBS1 is degradated by the *P. syringae* effector protein HopAR1 and the CC-NB-LRR protein RPS5 detects this degradation (Shao *et al.*, 2003; Swiderski and Innes, 2001).

It is important to underline that direct and guard type recognitions probably represent two ends of a spectrum with many intermediates (Rafiqi *et al.*, 2009). Caplan and co-workers (2008) showed that the tobacco N protein and the tobacco mosaic virus p50 helicase protein interact through a non-specific indirect mechanism mediated by the chloroplastic sulfurtransferase protein NRIP1. Interestly, although the *Arabidopsis* RRS1-R protein and its corresponding Avr factor PopP2 physically associate, RRS1-R/PopP2 recognition also requires another host protein, the cysteine protease *R*esponsive to *D*ehydratation (RD19) (Bernoux *et al.*, 2008). In addition, the *Arabidopsis* RIN4 protein, located in the plasma membrane, is influenced by three different *P. syringae* effectors, and associates *in vivo* with two NB-LRR proteins. Following the interaction with two effectors, AvrRpm1 and AvrB, RIN4 is phosphorylated and activates the RPM1 NB-LRR protein (Mackey e*t al.*, 2002). The third effector, AvrRpt2, is a cysteine protease activated inside the host cell, that eliminates RIN4 by cleaving it at two sites. This cleavage activates the RPS2 NB-LRR protein (Axtell e*t al.*, 2003; Mackey *et al.*, 2003). RIN4 interacts also with the GPI-anchored NDR1 protein that is required for the functionality of both RPM1 and RPS2 plant desease resistance genes (Jones and Dangl, 2006).

The recognition mechanism proposed by the guard model supports, therefore, the ability of a limited number of NB-LRR proteins to recongise a multitude of pathogen effectors, by focusing on the more limited number of potential host protein targets (Dangl and Jones, 2001). In some cases, a guardee does not play any important role in the absence of the receptor and its interaction with Avr is not associated with virulence and, consequently, its presence in the host cell does not enhance pathogen fitness. In order to explain this phenomenon, a new model of plant-pathogen interaction has recently been proposed with the name of "decoy"

model (Fig. 1.4C). According to it, after interacting with pathogens, plants generate specific proteins, similar to those targeted by pathogen effectors, that bind the effectors and mediate the recognisement by R proteins. Unlike the usual targets of effectors, the "operative target" in this model, these decoy proteins do not perform any function in a cell when R proteins are absent; however, through functional competition with the operative targets in binding pathogen effectors, they can reduce pathogen virulence and fitness (Glowacki *et al.*, 2010; van der Hoorn and Kamoun, 2008). Two hypotheses for the evolution of the "decoy" proteins have been proposed: the first implies that they represent a result of a modification and loss of the previous function of operative targets; while, according to the second hypothesis, other molecules, unrelated to resistance mechanisms, underwent the process of specific molecular mimicry. These molecules might posses some structural resemblances to the operative targets. It was proved that resistance of *S. lycopersicum* to *P. syringae* results from the presence of Pto kinase and a NB-LRR protein called Prf. It seems that interactions between the AvrPto effector, Pto and Prf occur according the decoy or guard model. In the first case, Pto is the decoy target of the AvrPto and mimics the intracellular kinase domains of PRRs, which are the operative targets of AvrPto. The interaction between Pto and AvrPto activates Prf. According to the guard model, Pto is an inhibitor of Prf and the binding with AvrPto deactivates its kinase activity, leading to the activation of Prf (Rafiqi *et al.*, 2009; Xing e*t al.*, 2007).

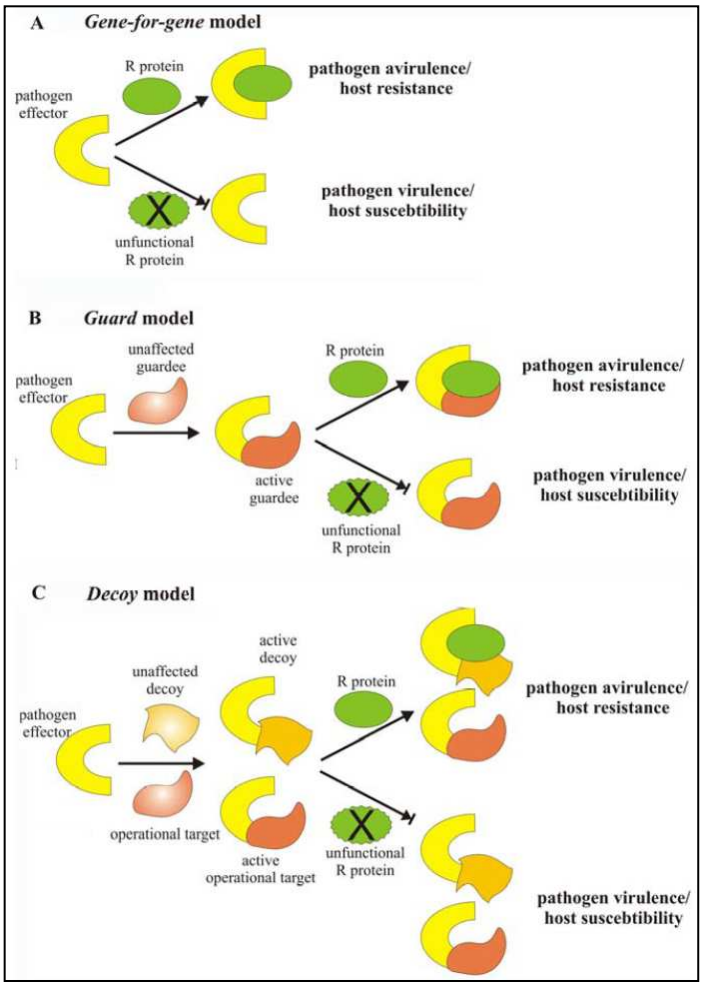


Fig. 1.4 Models of plant pathogens recognition (Glowacki *et al.*, 2010).

At this point, pathogen effector can be classified in two groups: MAMPs or PAMPs that are recognized by direct interactions with host receptors, whereas *M*icrobe-*I*nduced *M*olecular *P*atterns (MIMPs) when they generate a disturbances in the structure or function of host cells recognized by other receptors (Caplan *et al.*, 2008; Bittel and Robtzek, 2007; Mackey *et al.*, 2006).

NB-LRR proteins are found in a different cellular compartements. A well-studied example is the *Arabidopsis* TIR-NB-LRR RRS1-R protein that shows a NLS (*N*uclear *L*ocalization *S*ignal) motif; however its nuclear localization is dependent on the presence of its effector, which carries a functional NLS (Deslandes e*t al.*, 2003). Tobacco N and barley Mla10 proteins localize to the cytoplasm and nucleus. Nuclear localization of both this proteins is required for the induction of ETI (Burh-Smith *et al.*, 2007; Shen e*t al.*, 2007). Even the nuclear localisation of the *Arabidopsis* RPS4 protein, conferring resistance to *P. syringae* upon recognition of

AvrRps4, has been similarly found to be necessary for disease resistance (Wirthmueller *et al.*, 2007). Neverthless, only a few plant NB-LRR proteins are localized in the nucleus and many cloned R proteins do not carry an obvious NLS motif, but can be driven in this cellular compartement thank to the interaction with other cellular proteins (Rafiqi *et al.*, 2009).

Besides nucleocytoplasmatic localization, some R proteins have been found connected with membranes. For example, *Arabidopsis* RPM1 protein is periphally associated with the plasma membrane (Boyes e*t al.*, 1998). Another *Arabidopsis* TIR-NB-LRR protein, RRP1A, conferring resistance to the oomycete *Hyaloperonospora parasitica*, is targeted to the ER and golgi membranes (Weaver *et al.*, 2006).

The diverse localizations of the NB-LRR proteins could reside in the different locations of pathogen effectors and their cellular targets. However, this is not always the rule. Flax resistance proteins L6 and M associate with golgi bodies and plasma membrane, respectively, while their corresponding Avr proteins, AvrL567 and AvrM are nucleocytoplasmatic (Rafiqi e*t al.*, 2009). Another explanation for the different localization patterns would be the complete loss of entire domains of the NB-LRR proteins that rapidly evolve under diversifying pressure (Meyers *et al.*, 2003).

1.2.3 R proteins-mediated signalling pathways

Considering that the activation of defence responses, particularly the HR, requires significant costs to the plant, resistance mechanisms must be tightly regulated to prevent inappropriate signaling. Neverthless, this must be balanced against the need to induce a rapid and strong response in presence of pathogens (Rafiqi *et al.*, 2009).

As described in Par. 1.2.1.2, in the absence of an elicitor, R proteins exist in an inactive conformation that can perceive pathogen signals. In some cases, this conformation depends on association with chaperone protein complexes that facilitate the intramolecular interactions and conformational changes associated with transitions between the active and inactive signaling states (Rafiqi *et al.*, 2009). SGT1 (*S*uppressor of *G*2 allele of SKP1), HSP90 (*H*eat *S*hock *P*rotein 90) and RAR1 (*R*equired for ML*A*-dependent *R*esistance 1) are the major chaperones

associated with R proteins in plants as diverse as *Arabidopsis*, barley and tobacco (Azevedo e*t al.*, 2007; Hofius *et al.* 2007; Boter e*t al.*, 2007; Schultze-Lefert, 2004) and are thought to form a complex mediating the folding of R proteins and/or their incorporation in functional complexes (Shirasu and Schulze-Lefert, 2003). Interestingly, these requirements seem to be conserved across the *phyla*. The mammalian *N*OD-*L*ike *R*eceptors (NLRs) are NBS-LRR proteins involved in animal innate immunity and the functionality of many of them is associated to the formation of multiprotein complexes with SGT1, HSP90 and CHP-1, a mammalian CHORD-I/CHORD-II homologue of RAR1 (Mayor *et al.*, 2007; Staal and Dexelius, 2007).

Interestingly, specific mutations in *HSP90* suppress a loss-of-function mutation in *Rar1* and restore the accumulation and activity of *Arabidopsis* R proteins (Hubert *et al.*, 2009). It was observed that SGT1 interacts with a co-chaperone of HSP90, HSC70, and that the suppression of *HSC70* is induced during plant-pathogen interactions, while its overexpression leads to a partial loss of resistance. Due to these observations, it has been suggested that SGT1 may be a bridge between HSP90 and HSP70 (Noel *et al.*, 2007).

R proteins signal transductions involves two partially independent signaling: the EDS1- and the NDR1-dependent pathways (Fig. 1.5; Glowacki *et al.*, 2010). The EDS1 (*E*nhanced *D*isease *S*usceptibility 1) protein shows homology to eukaryotic lipases and is a mediator in the signaling transduction triggered by most TNL proteins (Fig. 1.5). It also plays a key role in the regulation of plant response to abiotic stresses (Hu *et al.*, 2005; Wiermer *et al.*, 2005; Falk *et al.*, 1999). Null *eds1* mutants of *A. thaliana* are not able to generate defence responses even in presence of mutations auctoactivating TIR-NB-LRR proteins (Bartsh e*t al.*, 2006). Contrarly, most CNL trigger signaling pathways are mediated by the NDR1 (*N*on-race-specific *D*isease *R*esistance 1) (Fig. 1.5). In fact, mutations in the *NDR1* gene suppress a subset of CC-NBS-LRR-dependent resistances (Aarts *et al.*, 1998). Although the NDR1 mode of action is far from being well known, a few examples of direct interaction of this protein with “guardee” as well as indirect interactions with R proteins have been discovered. It was found that the cytosolic N-terminal domain of NDR1 interacts with RIN4 and is guarded by RPM1 and RPS2 (Day *et al.*, 2006; 2005).

The fact that some R proteins (e.g. RPP7, RPP8 and RPP13) act through both EDS1 and NDR1 pathways suggests that these two signaling transductions are not completely independent to each other (Kuang *et al.*, 2004).

In addition to local resistances and HR, defence responses against biotroph pathogens can also lead to the long lasting *S*ystemic *A*cquired *R*esistance (SAR), a form of systemic immunity that can potentiate tissues against subsequent attack by the same or other pathogens (Durrant and Dong, 2004). SAR is dependent on *S*alicilic *A*cid (SA) that influence the activity of NPR1 which, in turn, modulates the systemic responce interacting with the WRKY and TGA transcription factor families (Fig. 1.5, Johson *et al.*, 2008).

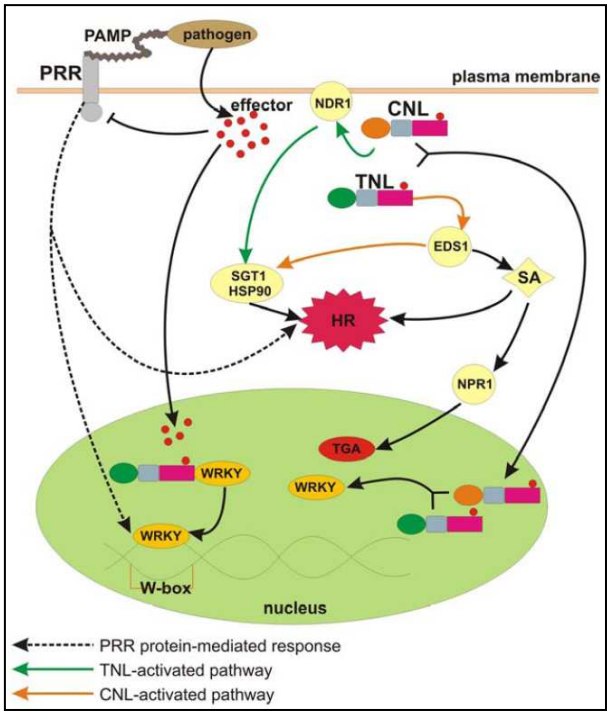


Fig. 1.5 Schematic representation of EDS1- and NDR1-dependent pathways. (Glowacki *et al.*, 2010).

After R proteins activation, the first cell responses include a rapid burst of reactive oxygen species (ROS) that may function as signalling molecules and/or executioners of pathogens (Torres e*t al.*, 2006) and lead to an increase in oxidative reactions (other ROS and production of nitric oxide) and transmembrane ion flux (especially Ca^{2+}, K^{+} and H^{+}). This results in the induction of cross-linking of phenolics with cell wall components and reinforcement of the plant cell walls by depositing callose and lignin, as well as the activation of signal cascades mediated by proteins kinase such

as MAPK (*M*itogen *A*ctivate *P*rotein *K*inase-MAPK) and G protein, that determine a transcriptional reprogramming including the induction of genes encoding antimicrobial proteins (defensins), antimicrobial secondary metabolites (phytoalexins) or *P*athogenesis *R*elated (PR) proteins (*e.g.* chitinases and glucanases) that form a protective barrier against pathogens, and often genes encoding factors involved in the HR. Amplification of initial responses accours *via* signals including ROS, lipid peroxides, *B*enzoic *A*cid (BA), *J*asmonic *A*cid (JA), *ET*hylene (ET) and in particular SA which can lead to the SAR. A cross-talk between all the pathways coordinates the resistance response (Figure 1.6; Buchanan *et al.*, 2000).

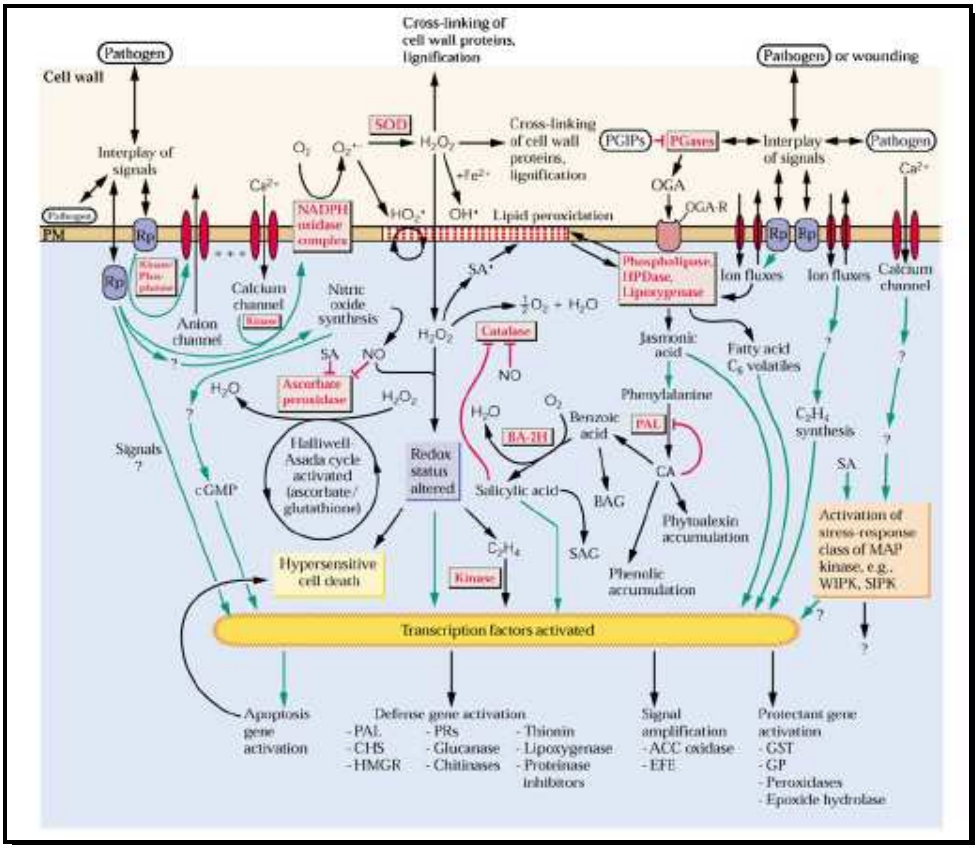


Fig. 1.6 Signal transduction in plant defence responses. A green arrow indicates positive interactions, and a red block negative ones. ACC=1-aminocyclopropane-1-carboxylic acid; BAG=benzoic acid glucoside; BA-2H=benzoic acid-2 hydroxylase; CA=cinnamic synthase, EFE=ethylene-forming enzyme, GP=glutathione peroxidase, GST=glutathione S-transferase, HMGR=3_-hydroxy-3-methyl-glutaryl-CoA reductase; HPDase=hydroxyperoxide dehydrase; OGA and OGA-R=oligogalacturonide fragments and receptor; PAL=phenylalanine ammonia.lyase; PGases=polygalacturonases; PM=plasma membrane; SA•=salicylic acid radical, SAG=salicylic acid glucoside, SIPK=salicylic acid-induced protein kinase, WIPK=wound-induced protein kinase, MAP=mithogen associated pathogen (Buchanan *et al.*, 2000).

At the end of defence responses, R protein signalings must be inactivated. Hofius and co-workers (2007) have proposed a role for the ubiquitin proteasome pathway as modulator of *R* gene-mediated resistance by eliminating R proteins and/or their interactors after the triggering of signal transductions. In *Arabidopsis*, the RING finger E3 ubiquitin ligases influence the RPM1- and RPS2-dependent HR and loss-of-function alleles of *Pub17*, encoding for an U box E3 ligase, are impaired in RPS4 mediated resistance (Yang *et al.*, 2006).

1.3 Barley leaf stripe disease

Barley (*Hordeum spontaneum*) leaf stripe disease is caused by the seed-transmitted hemibiotrophic fungus *Pyrenophora graminea* (anamorph *Drechslera graminea*) that causes a monocyclic strictly seed borne disease, with a teleomorph that is rarely seen in nature. The fungus survives as mycelium in the pericarp, the hull and the seed coat, but not in the embryo. Infection starts during germination when the mycelium, living on the pericarp of infected seeds, penetrates the coleorhiza and, from there, colonizes the plant starting from the root tips. Fungal hyphae grow intercellularly from the coleorhiza to the scutellum and the roots, or to the scutellar node, where infection of the plantlet starts. During this first colonization phase *P. graminea* behaves as a biotroph, but without forming appressoria, and degrades host cell walls using hydrolytic enzymes without causing cellular necrosis (Haegi *et al.*, 2008; Hammounda *et al.*, 1988; Platenkamp, 1976). Once infection spreads into the young leaves, growth switches to a necrotrophic phase with the production of a host-specific glycosyl toxin (Bulgarelli e*t al.*, 2010; Haegi and Porta-Puglia, 1995) that causes initially small, chlorotic, elongate yellow spots which develop into longitudinal dark brown necrotic stripes that extend the full length of the leaf sheath and blade, between leaf veins. Lesions usually coalesce leading to leaf death followed by splitting and fraying of the leaves. Spores produced on the infected leaves spread to infect nearby plant spikes (Fig. 1.7; Biselli e*t al.*, 2010; Bulgarelli *et al.*, 2010; Valè *et al.*, 2003).

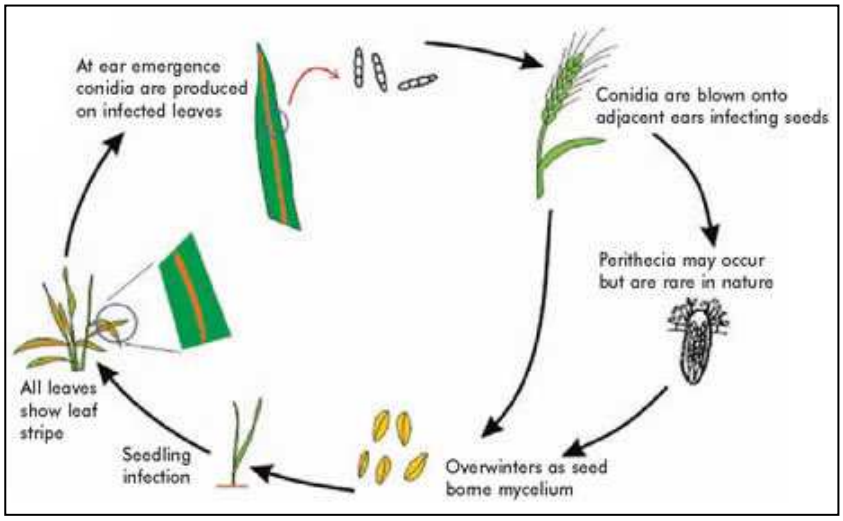


Fig. 1.7 Life cycle of *Pyrenophora graminea* (Cereal disease ancyclopedia-HGCA).

Infection of seeds can occur at any stage of development, from before head emergence through to soft dough stage, and symptoms may be visible at the first leaf stage, although more often they appear at the 4^{th}-5^{th} leaf stages. Infected plants are often stunted and head may become twisted, blighted or fail to emerge at all. Grain shrivelling and discolouration occurs where symptoms are severe (Valè *et al.*, 2003). The disease is particularly acute in Nordic countries (spring sowing) and in the Mediterranean's winter barley districts, because the establishment of plant infection is related to growing conditions of plantlets: plants kept at high temperatures (around 25°C) during germination frequently escape the infection by growing too fast; while plants kept at temperature below 12°C during germination allow more time for infection and the invading hyphae have better chances to growing through the coleorhiza and the scutellar node (Biselli *et al.*, 2010; Platekamp, 1976).

The yield losses due to *P. graminea* are correlated to degree of contamination of the seeds (Porta-Puglia *et al.*, 1986) and although in conventional farming systems the disease is controlled by chemical seed dressing, it has been calculated that when the percentage of infected seeds is high (over 30%), seed treatment is not effective to obtain acceptable yield, unless the variety has a substantial level of resistance (Delogu *et al.*, 1995). Moreover, the most severe yield losses are associated to organic farming systems which are important in many countries (Biselli *et al.*, 2010; Mueller *et al.*, 2003; Delogu *et al.*, 1995).

A variation in pathogenicity among different fungal isolates on the same genetic material has been reported, and the selective pressure by the pathogen strains on the host population may explain the existence of different resistance genes (Biselli e*t al.*, 2010). Neverthless, at the moment only two *Rdg* (*R*esistance to *Drechleslera graminea*) genes, *Rdg1a* and *Rdg2a*, are known. These genes cause hyphal degeneration in the basal part of the coleorhizae and prevent stripe symptoms from appearing on leaves of young or old plants (Bulgarelli *et al.*, 2004). *Rdg1a* confers resistance to *P. graminea* isolate *Dg5* and has been mapped to the long arm of chromosome 2H, using a segregating population represented by 103 *R*ecombinant *I*nbred *L*ines (RILs) of the cross L94 (susceptible) X Vada (resistant) and 194 RILs of the cross Arta (susceptible) X *H. spontaneum* 41-1 (resistant) (Biselli *et al.*, 2010). To date, this gene has not been cloned, but an *Rdg1a* syntenic interval with the rice chromosome arm 4L was identified. Although this region did not reveal any sequences strictly belonging to the *Nbs-LRR* genes, three genes coding for RLPK (*R*eceptor-*L*ike *P*rotein *K*inase) and a gene coding for a NB domain, were identified together with a homolog of the barley powdery mildew resistance gene *Mlo*. Three (out of five) homologs of these genes were mapped in the *Rdg1a* region in barley and the *mlo* homolog map position was tightly associated with the QTLs LOD score peak in both populations (Biselli *et al.*, 2010).

1.3.1 The barley *Rdg2a* locus

The barley *Rdg2a* gene is a mono-mendelian dominat gene identified for the first time in the French winter six-rowed barley cultivar *Thibaut* and confers immunity to at least three different *P. graminea* monoconidian isolates, including the most widespread and virulent Italian isolate *Dg2*, but it is overcome by isolate *Dg5* (Tab. 1.2; Biselli *et al.*, 2010; Bulgarelli e*t al.*, 2010; Gatti *et al.*, 1992).

Barley genotype	Leaf stripe isolate							
	Dg1	Dg2	Dg4	Dg5	Dg10	Dg12	Dg19	Dg23
NIL3876-*Rdg2a*	9[a]	0	68	95	40	8	0	60
Mirco-*rdg2a*	34	95	80	95	89	59	56	97

[a]Percentage of plants with leaf stripe symptoms

Tab. 1.2 Effectiveness of the leaf stripe resistance gene *Rdg2a*. *N*ear *I*sogenic *L*ines (NILs) with effective (*Rdg2a*) and non-effective (*rdg2a*) alleles at the *Rdg2a* locus were evaluated for resistance to nine different *P. graminea* monoconidial isolates (Bulgarelli a*t al.*, 2010).

Rdg2a is located distal on the short arm of barley chromosome 1 (7H) and is linked to the marker MWG2018 (Tacconi *et al.*, 2001). To mine the *Rdg2a* diffusion among resistant barley genotypes, the allelic composition at the MWG2018 locus of 19 resistant/susceptible barley cultivars and 150 barley accessions (originating from very different barley cultivation districts and belonging to the *B*arley *C*ore *C*ollection (BCC), http://barley.ipk-gatersleben.de) were analysed (Arru *et al.*, 2003b). Only in five resistant varieties the same MWG2018 allele of Thibaut was found; the observation that four of them (Rebelle, Haruna Nijo, Galleon and Acuario) showed the same pattern of resistance against isolates *Dg2* and *Dg5* of Thibaut raises the possibility that resistance to leaf stripe in these genotypes is governed by the same resistance gene. These cultivars represent very different barley genetic backgrounds: Thibaut and Rebelle are French six-rowed winter cultivars, while the two-rowed cvs. Acuario, Haruna Nijo and Galleon derived from Chile, Japan and Australia, respectively. These findings suggest that *Rdg2a* is widespread in different regions around the world and is carried by both six-rowed and two-rowed genotypes (Arru *et al.*, 2003b).

Due to the resistance conferred by *Rdg2a* to the isolate *Dg2*, this gene has been used since years to improve leaf stripe resistance in barley breeding programs of the Italian public institutions. Moreover, the availability of the linked marker MWG2018 allowed a successfully applications of MAS (*M*olecular markers-*A*ssisted *S*election) for *Rdg2a* (Arru *et al.*, 2003b; Francia *et al.*, 2005; Valè *et al.*, 2005).

Despite the importance of genetic resistance to leaf stripe, the molecular mechanisms underlying *P.graminea*-barley interactions are not completely

understood. In young barley roots of Thibaut, a number of PR genes, encoding for thaumatin-like proteins, thionins, peroxidase, ß-(1,3)-glucanase as well as *R*ibosome *I*nactivating *P*roteins (RIPs) were found to have altered expression in response to infection. However, no clear differences were found in the induction kinetics of these PR genes comparing compatible (*P.graminea-Dg5* vs. Thibaut-*Rdg2a*) and incompatible (*P.graminea-Dg2* vs. Thibaut-*Rdg2a*) interactions. This suggests a generic defence role in the host response for these genes, most likely a PAMPs/MAMPs-mediated response, rather than an *R* gene-mediated resistance (Valè *et al.*, 1994; 1995).

P. graminea isolate *Dg2* expressing the ß-glucuronidase (GUS) reporter was used to follow the penetration of the pathogen inside germinating barley seeds and the colonization of host tissues. Histochemical analysis showed that in susceptible cultivars the fungus invades the entire embryo and the coleoptiles (Fig. 1.8B), whereas in resistant cultivars it is restricted to the scutellar node and the basal region of provascular tissue (Fig. 1.8A; Haegi *et al.*, 2008; Aragona and Porta-Puglia, 1999).

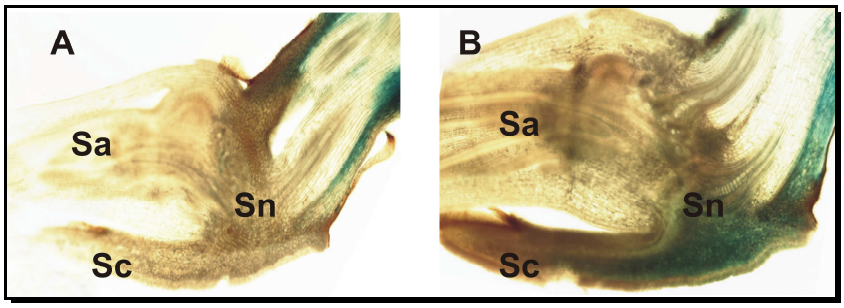


Fig. 1.8 Sections of barley embryos challenged by *P.graminea Dg2*-GUS observed under bright field illumination. **A** Resistant variety in which the colonization of the fungus (blue coloration) is restricted to the scutellar node. **B** Susceptible variety in which the fungus by-pass the scutellar node and diffuses in the scutellum. Sa=Shoot apex; Sc=scutellum; Sn=scutellar node. The sections were taken at the same time point after infection.

Interensingtly, Haegi and co-workers (2007) found that *Rdg2a*-immune response was associated with cell wall reinforcement through accumulation of phenolic compounds and enhanced transcription of genes involved in ROS production and detoxification/protection, without an apparent localized PCD.

To investigate the molecular basis of the *Rdg2a*-based resistance, a high resolution genetic map of the *Rdg2a* locus was constructed using a F2 population of 1,400

plants, derived from the cross between the varieties Thibaut-*Rdg2a* and Mirco-*rdg2a*. This map comprised several markers developed by using sequences conserved among plant disease resistance genes (*R*esistance *G*ene *A*nalogues-RGAs) (Leister *et al.*, 1999) and molecular markers developed by using the sinteny relationship between barley and rice in the genomic region of the *Rdg2a* locus. The markers developed from rice sequences allowed delimitation of the *Rdg2a* syntenic interval to a contig of 115 kbp in rice chromosome 6 (Fig. 1.9; Bulgarelli *et al.*, 2004).

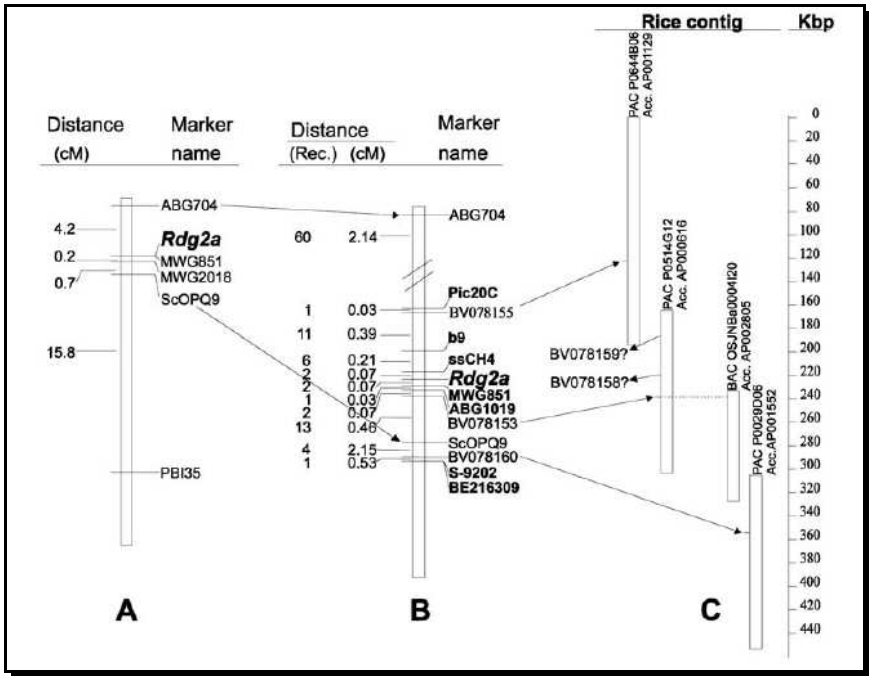


Fig. 1.9 Consecutive stages of high-resolution mapping at the *Rdg2a* locus. **A** The first map was constructed using a population of 218 F2 plants. Markers ABG704 and ScOPQ9 were used to screen a population of 1,400 F2 plants, and the resulting 93 recombinants were utilized to generate the second map (**B**). The two barley genetic maps are not drawn to scale. RGAs in **B** are shown in bold. **C** Three CAPS markers derived from rice ESTs (BV078155, BV078153, BV078160) enabled alignment to a rice physical contig of 453,648 kb comprising three PAC clones and one BAC clone. Arrows between **B** and **C** indicate the position of homologues present in rice. Question marks indicate loci which mapped to positions unlinked to *Rdg2a* in barley. Genetic distances (cM) as well as number of recombinants observed for specific intervals on the high resolution map are shown. Physical scale in rice is indicated on the right in **C**. Distances for markers proximal of ScOPQ9 were obtained using the 93 recombinants, although the observed recombination frequency was halved to correct for expected positive interference arising from selected recombination in the ABG704-ScOPQ9 interval (Bulgarelli *et al.*, 2004).

Analysis of the rice sequence failed to reveal any gene with similarity to characterized resistance genes, supporting either the hypothesis that *Rdg2a* encodes

for a novel type of resistance protein or that barley-rice synteny is disrupted in this region (Bulgarelli *et al.*, 2004).

To specifically identify the *Rdg2a* gene, more molecular markers at the *Rdg2a* locus were developed using an available Morex BAC library. Leaf stripe isolate *Dg2* is virulent on cv. Morex, indicating that this variety does not carry a functional *Rdg2a* allele, but the isolation of recessive alleles of *R* genes and markers using a Morex BAC libraries was previously efficient for the isolation of functional alleles at the *Mla* locus in barley (Halterman *et al.*, 2001; Zhou *et al.*, 2001; Wei *et al.*, 1999). Screening of the library with a probe derived from the CAPS marker MWG851, allowed the identification of BAC clones 146G20, 244G14 and 608H20 that were subjected to end sequencing. The 146G20 and 608H20 clones were also sequenced through a low-pass (0.3-fold) shotgun method and nine additional CAPS, dCAPS or RFLP markers were identified (Fig. 1.10A; Bulgarelli *et al.*, 2010). Two of these (146.60-1-2 and 146.9-5-6) showed complete linkage with *Rdg2a* in the high-resolution mapping population of 1,400 F2 plants and were tested on the three BAC clones, allowing the markers to be located to sections of the contig (Fig. 1.10B). The estimated size of the 146G20 insert was about 140 kbp. Markers 146.1F-1R and 146.4F-3R mapped 0.32 cM apart, indicating a genetic to physical ratio of about 440 kb per cM in this *Rdg2a* interval (Bulgarelli *et al.*, 2010).

To clone the region containing the *Rdg2a* gene, a cosmid library of Thibaut was constructed and screened using markers 146.9-5-6 and 608.32-3-4 (Fig. 1.10A), leading to the identification of the clones 95-3-3 and 17-1-1. Analyses of these two clones with other PCR markers from the region indicated that they spanned the *Rdg2a* interval bounded by the closest flanking genetic markers (Fig. 1.10C). The two cosmid inserts, overlapping for 5.9 kb, were sequenced, providing a contiguous sequence of 72,630 bp. BLASTX analyses showed that this region contains three genes similar to plant *R* genes encoding NB-LRR proteins (AC: HM124452). Auto Predgeneset tool of RICEGAAS software (http://ricegaas.dna.affrc.go.jp/; Sakata *et al.*, 2002) was used to predict these genes that were named *Nbs1-Rdg2a*, *Nbs2-Rdg2a* and *Nbs3-Rdg2a* (Fig. 1.10C; Bulgarelli *et al.*, 2010).

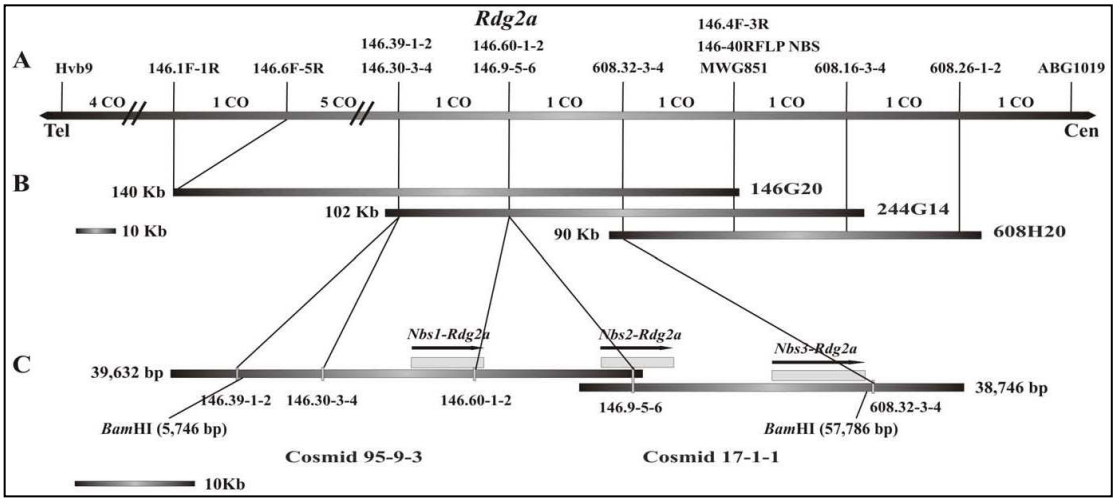


Fig. 1.10 Genetic and physical maps of the *Rdg2a* locus. **A** Genetic map of *Rdg2a*. Crossovers identified in the 1,400 F_2 plants from a cross between Thibaut (resistant) and Mirco (susceptible) (Bulgarelli e*t al.*, 2004) cultivars are shown at the top (CO). Orientation is indicated by Tel (telomere) 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 (Bulgarelli *et al.*, 2010).

Genomic DNAs of cvs. Thibaut, Morex, Golden Promise (a susceptible variety), Mirco and its resistant *N*ear *I*sogenic *L*ine NIL3876 were digested with BamHI. Following this, DNAs were screened with probes specific for each of the *Rdg2a* genes in Southern blot analyses. Only one fragment of about 50 kpb was detected in the resistant Thibaut and NIL3876 (Fig. 1.11), in agreement with the 52 kbp fragment size predicted from the sequence assembly (Fig. 1.10C). On the other hand, in susceptible genotypes, either three fragments (Mirco and Golden Promise) or a single fragment of about 20 kpb (Morex) were found (Fig. 1.11), suggesting the presence of significative rearrangements and in particular, one or more deletions at the Morex *rdg2a* locus (Bulgarelli *et al.*, 2010).

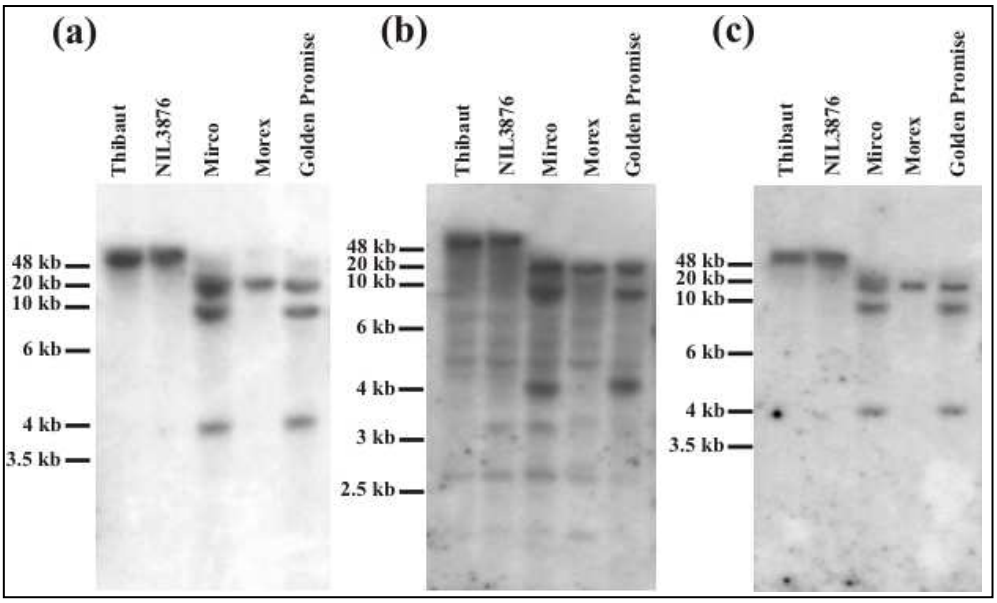


Fig. 1.11 Southern blot analyses of the *Rdg2a* genes using specific probes for the three genes (*Nbs1-Rdg2a* **(a)**, *Nbs2-Rdg2a* **(b)** and *Nbs3-Rdg2a* **(c)**) and BamHI digested genomic DNA from cvs. Thihbaut (resistant), Morex (susceptible), Golden Promise (susceptible), Mirco (susceptible) and its resistant NIL, NIL3876.

In the present work, the identification and functional characterization of the *Rdg2a* gene were carried out. The analysis of the transcript structure of the three *Rdg2a* candidates allowed the exclusion of the *Nbs3-Rdg2a* gene, encoding for truncated proteins. The sequencing of *Nbs1-rdg2a* and *Nbs2-rdg2a* in the susceptible cv. Mirco revealed significative rearrangements in the putative promoter regions of these genes that, most likely, abolished their transcription. Rearrangements are also present in the hypothetic *Rdg2a* locus of cv. Morex, which showed two deletions that eliminated most of the Morex *Nbs1-Rdg2a* coding sequence and generated an hybrid gene between the *Nbs1-Rdg2a* putative promoter region *plus* 5' end of the gene with the *Nbs2-Rdg2a* coding sequence. Real-time PCRs showed that *Nbs1-Rdg2a* expression does not vary in presence of *P. graminea* isolate *Dg2*, while *Nbs2-Rdg2a* transcript level increased in the first stages of the infection; however the aboundance of the mRNA of this gene is lower than that of the *Nbs1-Rdg2a* transcript. Most interestingly, when the susceptible variety Golden Promise was transformed with the two candidates, under the control of their native promoters, only transgenic plants carrying *Nbs1-Rdg2a* were completely resistant to isolate *Dg2*, thus confirming that this gene is *Rdg2a*. TUNEL hystological analyses, conducting on section of barley embryos, demonstrated that the *Rdg2a*-mediated resistance does not involve HR at the level of infected cells.

2. Materials and methods

2.1 Barley leaf stripe causal agent *Pyrenophora graminea*

In this study, two highly virulent isolates of the barley leaf stripe causal agent *Pyrenophora graminea*, *Dg2* and *Dg5*, were used. In particular, *Dg2* is the most virulent isolate of a previously described collection of monoconidial isolates (Gatti *et al.*, 1992). Fungi were grown on Petri dishes on PDA (*P*otato *D*extrose *A*gar) medium (Liofilchem, Teramo, Italy) at 19°C for 10 days in the dark, then stored at 4°C.

2.2 Plant materials

cv. Thibaut: barley variety carrying the resistance gene *Rdg2a* and fully resistant to *Pyrenophora graminea* isolate *Dg2*, but susceptible to isolate *Dg5* (Arru *et al.*, 2003).

cvs. Mirco and Golden Promise: barley varieties without a functional allele of the *Rdg2a* gene and fully susceptible to *P. graminea* isolates *Dg2* and *Dg5* (Arru *et al.*, 2003).

cv. Morex: barley variety partially susceptible to *P. graminea* isolates *Dg2* and fully susceptible to isolate *Dg5* (Arru *et al.*, 2003).

NIL 3876: near isogenic line in Mirco genetic background generated by an initial cross between Thibaut and Mirco followed by six backcrosses with cv. Mirco and simultaneous selection for leaf stripe resistance (Tacconi *et al.*, 2001); this line contains a functional allele of the *Rdg2a* gene and is fully resistant to *P. graminea* isolate *Dg2*, but susceptible to *Dg5*.

Transgenic lines: transgenic barley lines carrying *Nbs1-Rdg2a* (lines T6) or *Nbs2-Rdg2a* (lines T7) from cv. Thibaut. These plants were generated by *Agrobacterium tumefaciens*–mediated transformation of immature embryos derived from *Hordeum vulgare* ssp. *vulgare* (barley) cv. Golden Promise as described by Bieri *et al.* (2004). The presence of the transformed DNA fragments in T_0 plants was checked by PCR with gene-specific primer pairs. T_1 progenies were bred through self-pollination of

T_0 plants. The transgene copy number was evaluated by Southern hybridization analysis (Bulgarelli *et al.*, 2010).

cvs. Rebelle, Proctor, Alf and Onice: barley varieties resistant to *Pyrenophora graminea* isolates *Dg2* and *Dg5* (Arru *et al.*, 2003).

cv. Diadem: barley variety fully resistant to *Pyrenophora graminea* isolate *Dg2* (Arru *et al.*, 2003).

cvs. Nudinka and Jaidor: barley varieties highly susceptible to both the *Pyrenophora graminea* isolates (Arru *et al.*, 2003).

cvs. Haruna Nijo, Galleon and Acuario: barley varieties carrying the resistance gene *Rdg2a* and fully resistant to *Pyrenophora graminea* isolate *Dg2*, but susceptible to isolate *Dg5* (Arru *et al.*, 2003).

cvs. Rika, Bulbul, Triumph, Imber, Optic, Ansis, Gitane, Bonus, Ketos, Grete, Franka, Marado and Federal: barley varieties whose resistance level towards *Pyrenophora graminea* is unknown.

2.3 Infection of the seeds with the leaf stripe isolates

Seeds were surface-sterilized in 70% ethanol for 30s and then in 5% sodium hypochlorite for 20min; seeds were then extensively rinsed in distilled water prior to inoculation with different leaf stripe isolates using the 'sandwich' technique (Pecchioni *et al.*, 1996).

2.4 Analysis of the *Rdg2a* candidates

Using a map-based cloning approach, three *N*ucleotide-*B*inding, *L*eucine-*R*ich *R*epeat (NB-LRR) encoding genes were identified at the *Rdg2a* locus; these genes were named as *Nbs1-Rdg2a*, *Nbs2-Rdg2a* and *Nbs3-Rdg2a*. The first two genes were characterized during a previous PhD thesis (Par. 1.3.1, Introduction; Bulgarelli *et al.*, 2010). In the present work, a structural characterization of the third member (*Nbs3-Rdg2a*) of the gene family and a functional characterization of the other two genes were performed.

2.4.1 Sequencing of *Nbs3-Rdg2a* cDNA from NIL3876

2.4.1.1 *R*everse *T*ranscription-PCR and *R*apid *A*mplification of *c*DNA *E*nds (RACE)

*R*apid *A*mplification of *c*DNA *E*nds (RACE) was performed using the GeneRacer kit (Invitrogen, Carlsbad, CA USA) using 250ng of DNaseI (DNA-*free*™ Kit, Ambion) treated poly(A)RNA extracted from NIL3876 embryos inoculated for 7 days with leaf stripe isolate *Dg2* and from embryos grown for 7 days on sterile moist filter paper (control), according to Baldi *et al.* (1999). To obtain the 5' and 3' ends of *Nbs3-Rdg2a* cDNA, 2 rounds of PCR were performed using the specific primers (Gene Racer 5' primer, GeneRacer 5' nested primer, GeneRacer 3' primer, GeneRacer 3' nested primer) supplied by the kit, and specific primers for the gene (Tab. 2.1).

cDNA sequences internal to RACE products were obtained by amplification of overlapping fragments, executing nested PCRs with gene specific primers (Tab. 2.1) and Platinum® *Pfx* DNA Polymerase (Invitrogen).

Fragment	**Primers combination**	**Sequence**
5' fragment	GeneRacer 5' primer	CGACTGGAGCACGAGGACACCTGA
	Nbs3_11	TCAGGCATGTCACATCTTCCACTTAC
	GeneRacer 5' nested primer	GGACACTGACATGGACTGAAGGAGTA
	Nbs3_12	CTTCTGTTCTTGCTGGTCCAACAGTTT
Internal fragment I	Nbs3_33	GCTGAGGAGATGAGCGAGAAGAAGTC
	Nbs3_21	TGCAACTGTCGGCAGTCTATGAGC
	Nbs3_33	GCTGAGGAGATGAGCGAGAAGAAGTC
	Nbs3_35	CCAAGATACCTAGCAGACCTCACTGAC
Internal fragment II	Nbs3_36	AAGAGAGAACAATGGATTTAACACGGAA
	Nbs3_25	GTTGTCAGGTTATCCATCCTCTGTAAGAG
	Nbs3_36	AAGAGAGAACAATGGATTTAACACGGAA
	Nbs3_26	GAGATGCCGAGAGCCATATTACAGGGAT
Internal fragment III	Nbs3_44	GTCTGATAGCCCGAAATGCAAGAGTATCCC
	Nbs3_2	GCATCGTCTTACCAACTCCGGGCAATATTT
	Nbs3_30	CTCTTACAGAGGATGGATAACCTGACAAC

	Nbs3_4	TTCAGGCTCCTGCAGTGCAGCA
Internal fragment IV	Nbs3_1	TGGATCGCCTCCGCGTTCTGTATG
	Nbs3_45	GGCTTCTTTTGCATTCTCCCCACTCT
	Nbs3_37	AGGGGTCTCCGTGTGCTGCACTGC
	Nbs3_23	CGCAACTTCTGGCAATCCATGAGC
Internal fragment V	Nbs3_39	GCTCATGGATTGCCAGAAGTTGCG
	Nbs3_40	GGGTTTCCTCCTCCTCCTCATATGATG AAG
	Nbs3_39	GCTCATGGATTGCCAGAAGTTGCG
	Nbs3_41	CCCTTCAGGTAGTCACAATTTCTGAT
Internal fragment VI	Nbs3_32	GTTACCAAGCCTGGAGATGTGGGCAG AA
	Nbs3_13	CCTAATGCGTTTACGTGGAACAGAGG AGA
	Nbs3_43	GATGTGGGCAGAAAATAGTATGGGA GAG
	Nbs3_13	CCTAATGCGTTTACGTGGAACAGAGG AGA
Internal fragment VII	Nbs4_2	CACTTCATCATATGAGGAGGAGGAGG A
	Nbs4_3	CAACTCCGGGCACTCACTTATGCTT
	Nbs4_1	CTCTGAGGGAATTATGGATTTGGAA
	Nbs4_3	CAACTCCGGGCACTCACTTATGCTT
3’ fragment	Nbs3_43	GATGTGGGCAGAAAATAGTATGGGA GAG
	GeneRacer 3’ primer	GCTGTCAACGATACGCTACGTAACG
	Nbs3_42	AGCATAGTTGGAGCTCACAGTACTGC AGTC
	GeneRacer 3’ nested primer	CGCTACGTAACGGCATGACAGTG

Tab. 2.1 Primers used to obtain 5’ end, internal fragments and 3’ end of the *Nbs3-Rdg2a* cDNA.

The conditions for the first and the second rounds of PCR were:

Platinum® *Pfx* DNA Polymerase	1U
Pfx Amplification Buffer	1X
Pfx Enhancer Buffer	1.5X
dNTP mixture	0.3 mM each
$MgSO_4$	1 mM
Primer mix	0.3 µM each
Template DNA	4 ng
Autoclaved, distilled water	to 50 µl

The program of the touchdown PCR was:

94°C	2’	
94°C	30’’	6 cycles
60°C	35’’	
68°C	1’ per kb	
94°C	30’’	6 cycles
58°C	35’’	
68°C	1’ per kb	
94°C	30’’	26 cycles
55°C	35’’	
68°C	1’ per kb	
68°C	10’	
4°C	∞	

RACE products were purified from 1% agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega) and cloned into the Zero Blunt TOPO PCR vector (Invitrogen). After the transformation of One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen) cells, the selection with Ampicillin and plasmid purification by the Wizard® *Plus* SV Minipreps DNA Purification System (Promega), at least two independent clones for each PCR product were sequenced.

2.4.1.2 Sequencing procedures

Clones were sequenced using the Big Dye Terminator v3.1 Kit (Applied Biosystem, Foster City, CA USA) and ABI3130 Sequencer (Applied Biosystem, Foster City, CA USA). 250ng of Miniprep were utilized for each reaction. Each clone was sequenced by the use of M13 for (TGTAAAACGACGGCCAGT) and M13 rev (-29) (CAGGAAACAGCTATGACC) primers and, for the longest internal fragments, primers overlapping internal sequences of the PCR product (Tab. 2.2).

Fragment	**Primer**	**Sequence**
Internal fragment II	Nbs3_33	GCTGAGGAGATGAGCGAGAAGAAGTC
Internal fragment IV	Nbs3_4	TTCAGGCTCCTGCAGTGCAGCA
Internal fragment V	Nbs3_55	ATGCGTCGGCGAGCACCTTGCCG
	Cos 189	ACTCGGACGTACTTATTTATGTCT
Internal fragment VII	Nbs4_1	CTCTGAGGGAATTATGGATTTGGAA

Tab. 2.2 Primers used to sequence the longest internal fragments of *Nbs3-Rdg2a* cDNA.

Sequencing outputs were analyzed by the Sequencing Analysis Software v5.2 with KB Basecaller Software v1.2 (Applied Biosystem, Foster City, CA USA).
To assemble the sequences Vector NTI 9 software (Invitrogen, Carlsbad, CA USA) was used.

2.4.2 Sequencing of *Nbs1-rdg2a* and *Nbs2-rdg2a* alleles in the susceptible cultivar Mirco

Fragments of *Nbs1-rdg2a* and *Nbs2-rdg2a* were amplified from cv. Mirco genomic DNA, using *Nbs1* and *Nbs2*-specific primers, designed from cv. Thibaut genomic sequences of the two genes (Tab. 2.3).

Gene	Fragment	Primer combination	Sequence
Nbs1-rdg2a	Fragment I	Nbs1_17	CACCGCATCATGAAGAGAACTGATACAGGA
		D2_19	CCTTGCCGGCCACGCCGCGCACTAG
	Fragment II	D2_16	CTGTTCTTGTACATGCTGCAGCTTCC
		D2_17	TCGCAACTTCCGGCAATCCATTAG
	Fragment III	D2_13	GTTGCTACAGGTATCGGCATCACTAAGAGC
		D2_15	GGAACAGAGGAGAGCAAGTGGAAGTAC
	Fragment IV	Nbs1_15	CAGAACTGCCGCAGTGTAGTAGC
		Nbs1_19	GGTACCATCGATTCATGACGTTAGCAT
Nbs2-rdg2a	Fragment I	Nbs2_6	CACCGCAGAAGAATGCCTACAAAACCCTGA GTCC
		Nbs2_29	CAAGGTAAGGATTGAGGAGAGC
	Fragment II	Nbs2_34	GGGCGGCGACGAACAAGCAGCAGTAT
		Nbs2_16	TTCAACTTGTAACAGTCTATGAGC
	Fragment III	Nbs2_15	GCATTGCATTGCTCCCGCTCCCCTTCTCCAA
		Nbs2_22	GTGATCCGGACGATCCGGAGCTTCCG
	Fragment IV	Nbs2_23	TGTTGCCTCTGGACGCCCAGCAAACC
		Nbs2_5	CGGGCAGCCACGTATGCTAAAGG
	Fragment V	Nbs2_2	GGAGATTCAGGTCTGCCGCAGAGTG
		Nbs2_33	CAAGCAAGAGTCTAGCGCGTGAGG

Tab. 2.3 Primers used to amplify subsequent fragments of *Nbs1-rdg2a* and *Nbs2-rdg2a* in cv. Mirco.

PCR reactions were conducted using the following protocol:

Genomic DNA from Mirco leaves	40 ng
GoTaq®Flexi Buffer	1X
$MgCl_2$	2 mM
DMSO	5%
dNTP	0.2 mM each
Primer mix	0.3 mM each

GoTaq® DNA Polymerase	0.5 U
Sterile distilled H_2O	to 20 µl

and the following programme:

94°C	2’	
94°C	40’’	35 cycles
60°C	50’’	
72°C	1’ per kb	
72°C	10’	
4°C	∞	

After purification from 1% agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega), PCR products were directly sequenced using 2,5 ng each 100 bp of DNA. The same primers utilized for PCR reactions (Tab. 2.3) and primers overlapping internal sequences (Tab. 2.4) were used for sequencing.

Gene	**Fragment**	**Primer**	**Sequence**
Nbs1-rdg2a	Fragment I	Nbs1_1	CTTCACCGCGCTTACACAGTGCCA
		D2_11	CCTTACCAACGCCCAAATTTGTCG
		D2_20	CGATGAAGAGCAAAACCAGAGG
		Nbs1_11_1_17	GTGCTATTTCTGGTTTTCAA
		Nbs1_12_1_17	GGGGTACAGTTGCAAATAAA
		Nbs1_13_1_17	GAGAGGCTACATCTCAGATCTT
		Mirco1_22	GAAAGTAAGTAAATAGAAGGGG
		Mirco1_18	AGGAAGATAACAAGGTTGTT
		Mirco1_19	GCAAAAATGCCACCTGGCTC
		Mirco1_20	TTCCTTTTGCTTTTCCATTTACGTG
		Mirco1_21	ACTAGACTGCCCCTGTTCGTG
		Nbs1_14_1_17	GAACACGAGAAAATTGGATA
	Fragment II	Nbs1_9	ATTGGTCACATGTCGAAGCAAGCAAGTCGC
		Nbs1_11	GTAACATCGGGGATAAAGATGGAGGC
		Nbs1_20	CACCCTGTTCTTGTACATGCTGCAGCTTCC
		Nbs1_20Davide	TTCCCTTCCAAAGATCTGGGTAGTT
		Nbs1_21	CTATTACACGTGGCAATGCGG
		Nbs1_3	CAAATAAACCAAGTTGCAGGGGCC
		Nbs1_2	GGCCAACACAATGCTCTTAGTGATGCCGAT
		D2_5	GGAAATGACAACTGAATAAGAGGGCC
		Nbs1_4	CAGGCGACAGGTGTTTGTAGCTTA
		Nbs1_2	GGCCAACACAATGCTCTTAGTGATGCCGAT
	Fragment III	Cos189	AGCTTTACAAACCGACGGTT
		Nbs1_12	TCATCAGATCTCGCACGAACCGA
		D2_8s2	AGAAATATCACAATGGATGAGAA
		Nbs1_7	CCTCGGATGTTTAGCAGTTTGGA
		D2_2	CACCTTCTCTGCATCGTCTTTGC

	Fragment IV	D2_2	CACCTTCTCTGCATCGTCTTTGC
		D2_15	GGAACAGAGGAGAGCAAGTGGAAGTAC
		Nbs1_8	AGACTCACGCGTATGCCGATTCA
		Nbs1_6	GTCAGGTAAGGAGACTCACGCGT
		D2_9	CCATTGGTTATCACCTAATTTGTAT
Nbs2-rdg2a	Fragment I	Nbs2_30	TGAGGGTAGGCACACTGCACAC
		Nbs2_31	GAGATAATCAAAGGTGCGCCTCC
	Fragment II	Nbs2_36	CAACTTTGGGGCTTCGTTGAGGTG
		Nbs2_14	CACCTGTTTCCTGGCTCAATAGGAAGAC
		Nbs2_35	TCGACGAGTGCTTCTGCGGCCT
		Nbs2_19	CGGCCTCTATAATGCAGACCCTTGGAA
		Nbs2_12	GGCTTCGACTTGTGACAACAATGACG
		Nbs2_11	GCTAGCTTATGGGTTCCAAGGGTCTG
		Nbs2_18	CATATAGCAATGGTAAAGAGCAGG
	Fragment III	Nbs2_25	GTAATGTGGAAGAAGTGCTTCAATAT
		Nbs2_26	CGAAAAGTTGGAGATATGTGGGTATAT
	Fragment IV	Nbs2_24	CAGCTTGATCGGAAGCTCCGGATCG
	Fragment V	Nbs2_5	CGGGCAGCCACGTATGCTAAAGG
		Nbs2_10	TCTAACAGTCTTTACGTGGGACAGA
		Nbs2_32	AGTTTAGCAACTGCTCCTTGTAACCGCC

Tab. 2.4 Primers used for the sequencing of Mirco *Nbs1-rdg2a* and *Nbs2-rdg2a*.

The accession number assigned to *Nbs1-rdg2a* and *Nbs2-rdg2a* are HM124453 and HM124454, respectively.

2.4.3 Screening by PCR-based molecular markers to verify co-segregation of promoter rearrangements with the *Rdg2a* locus

To verify whether the differences between the promoter regions of Thibaut *Nbs1-Rdg2a versus* Mirco *nbs1-rdg2a* and Thibaut *Nbs2-Rdg2a versus* Mirco *Nbs2-rdg2a* cosegregated with the resistant phenotype, a screening using PCR-based molecular markers was performed on genomic DNA extracted from cvs. Thibaut and Mirco and from rare recombinants (231, 355, 407, 581, 604, 618, 741, 765, 844, 923, 1155 and 1845) identified in a high resolution mapping population, represented by 2.800 gametes and derived from a cross between the two genotypes. Primers overlapping sequences in the promoter regions of *Nbs1-Rdg2a* and *Nbs2-Rdg2a* were utilized (Tab. 2.5).

Gene	Primer	Sequence
Nbs1-Rdg2a	Nbs1_8	AGACTCACGCGTATGCCGATTCA
	Nbs1_15	CAGAACTGCCGCAGTGTAGTAGC
Nbs2-Rdg2a	Nbs2_6	CACCGCAGAAGAATGCCTACAAAACCCTGAGTCC
	Nbs2_29	CAAGGTAAGGATTGAGGAGAGC

Tab. 2.5 Primers used for the screening using PCR-based molecular markers of the population derived from a cross between Thibaut and Mirco, to verify co-segregation of promoter rearrangements with the *Rdg2a* locus.

PCRs were performed as described in Par. 2.4.2 and the amplicons were loaded on 1% agarose gel.

2.4.4 Expression analysis of the *Rdg2a* candidates

2.4.4.1 Semiquantitative RT-PCR

The assessment of *Nbs1-Rdg2a* and *Nbs2-Rdg2a* gene expression level was obtained in a two-step *R*everse *T*ranscription PCR (RT-PCR) process. Total RNA was extracted using TRIZOL® Reagent (Life Technologies), according to manifacturer's instructions, from barley embryos of Mirco and NIL3876 at 7 and 14 dai (*d*ays *a*fter *i*nfection) with *Pyrenophora graminea* isolate *Dg2* and after growth on sterile moist filter paper (control) at the same time points. Total RNA was also axtracted from non-inoculated leaves of 7 days old barley seedlings of cv. Mirco and NIL3876. After the analysis of the RNAs with the 2100 Bioanalyzer (Agilent), the quantification using spectrophotometer and treatment with DNaseI (DNA-*free*™ Kit, Ambion), 400 ng of each RNA were used for the initial RT reaction with the Superscript II reverse trascriptase kit (Invitrogen, Carlsbad, CA USA). For the second PCR reaction, 4 ng of first strand cDNAs, quantified by using fluorometer Qubit (Invitrogen, Carlsbad, CA USA), were used as templates. Each experiment was performed in triplicate. To enable the discrimination between the two *Rdg2a* candidates and other NB-LRR encoding genes, the RT-PCR primers were designed on two different regions of the LRR domains where several mismatches among the two genes allowed gene-specific amplification for the two genotypes. To confirm specific amplification, the amplicons were then sequenced using the same primers utilized in PCR reactions (Tab. 2.6) and following the protocol described in Par. 2.4.2. Barley *Actin* (AY145451.1) was used as positive RT-PCR control (barley

Actin primer fw: ATGTGGCCATCCAGGCAGTGCTTT, barley *Actin* primer rev: TGGTCTCATGGATTCCAGCAGCTTCC). PCRs were performed with:

$MgCl_2$	2 mM
dNTP	0.2 mM each
Primer mix	0.3 mM each
DMSO	5%
Go Taq® DNA Polymerase (Promega)	0.5 U
Go Taq® DNA Polymerase Buffer	1X
cDNA template	4 ng
Sterile, distilled water	to 20 µl

The PCR program was:

94°C	2’	
94°C	40’’	24 cycles for *Actin* and 30
60°C	50’’	cycles for the other two genes
72°C	2’	
72°C	10’	
4°C	∞	

Primers used are listed in Tab. 2.6.

Line	**Gene**	**Primers**	**Sequence**
NIL3876	*Nbs1-Rdg2a*	Nbs1_25	GATGAGCCTACAGATGTGGAAGAAGTGC
		Nbs1_26	GCTAAACATCCGAGGCTCTCCTACACTA
		Nbs1_27	TTTCATCATCCGAGGAGAAAACCCTTCCGC
		Nbs1_28	GCCGATTCACTTTGGGATGCCTATTCTCTC
	Nbs2-Rdg2a	Nbs2_3	TTTGTTATCTCCTTCAGAATCATGGGAG
		Nbs2_4	GAAGCACTTCTTCCACATTACAGGCC
		Nbs2_2	GGAGATTCAGGTCTGCCGCAGAGTG
		Nbs2_5	CGGGCAGCCACGTATGCTAAAGG
Mirco	*Nbs1-Rdg2a*	Nbs1_3_m	TGATTTGGGGCTGCCGAAGTCTGGT
		D2_7	TTTGTCAGGTAAGGAGACTCACGC
		Nbs1_3_m	TGATTTGGGGCTGCCGAAGTCTGGT
		Nbs1_6	GTCAGGTAAGGAGACTCACGCGT
	Nbs2-Rdg2a	Nbs2_2_m	GACGATTGATAACTGCCGCAGTGTA
		Nbs2_5	CGGGCAGCCACGTATGCTAAAGG
		Nbs2_7_m	TGGGTGGAGGACTGCATGAGCCTAA
		Nbs2_5	CGGGCAGCCACGTATGCTAAAGG

Tab. 2.6 Primers used for the semiquantitative RT-PCRs for *Nbs1-Rdg2a* and *Nbs2-Rdg2a* in the cv. Mirco and in NIL3876.

PCR products were loaded on 1% agarose gel.

2.4.4.2 *q*uantitative RT-PCR (qRT-PCR)

To examine whether the *Nbs1-Rdg2a* and *Nbs2-Rdg2a* transcription levels change during the infection, a two-step *q*uantitative (q)RT-PCR was performed. Total RNA was extracted by the use of TRIZOL® Reagent (Life Technologies) from barley embryos of NIL3876 at 7, 14, 18, 22 and 28 dai with *Pyrenophora graminea* isolate *Dg2* and from embryos grown on sterile moist filter paper (control) at the same time points. cDNAs were synthesized as described in Par. 2.4.4.1.

qRT-PCRs were performed in a real time PCR thermal cycler (7300 Real Time PCR System, Applied Biosystem) with:

cDNA	1 ng
Sybr GreenER qPCR SuperMix for ABI PRISM (Invitrogen)	10 µl
Primers mix	0.2 µM each
Sterile, distilled water	to 25 µl

Cycling conditions were:

50°C	2’	
95°C	10’	40 cycles
95°C	15’’	
60°C	1’	

The primers utilized were as for semiquantitative RT-PCR (Tab. 2.6). Barley *Actin* (AY145451.1) was used as the housekeeping normalizator (barley *Actin* primer fw: ATGTGGCCATCCAGGCAGTGCTTT, barley *Actin* primer rev: TGGTCTCATGGATTCCAGCAGCTTCC). Two biological replicates with eight technical replicates each were performed. Melting curve analysis was done after PCR to evaluate the presence of non-specific PCR products and/or primer dimers. qRT-PCR data were plotted as ΔRn fluorescence signal versus cycle number. The SDS 7300 absolute quantification software (Applied Biosystem) calculates the ΔRn using the equation ΔRn = (Rn+) – (Rn-), where Rn+ is the fluorescence signal of the baseline emission during cycles 6 to 13. An arbitrary threshold was set at midpoint of the log ΔRn versus the cycle number at wich the ΔRn crosses the threshold (Ct). The Ct was used to calculate the *F*old *C*hange (FC) in each infected sample with respect to the

expression level detected in corresponding sample in control conditions at the same time point (baseline) with the following formula:

$$FC = 2^{-\Delta\Delta Ct}$$

$\Delta\Delta Ct$ = (Ct target – Ct actin)infected sample – (Ct target – Ctactin)uninfected sample.

2.5 Analysis of transgenic plants

2.5.1 Production of transgenic plants

To generate constructs for transformation, DNA fragments of about 6 Kbp containing the coding sequence of the candidate genes with their native 5' and 3' regulatory sequences, were PCR amplified using Phusion HF Taq DNA Polymerase (New England Biolabs) from cosmid 95-9-3 (*Nbs1-Rdg2a*) and cosmid 17-1-1 (*Nbs2-Rdg2a*), derived from the cv. Thibaut. Amplification products were subcloned in pDONR201 (Invitrogen) and then transfer in the gateway *Agrobacterium tumefaciens* binary vector pWBVec8 (Invitrogen). These constructs were validated by comparing the insert sequences with those of the corresponding regions in the cosmid clones. Transgenic barley lines were generated by *A. tumefaciens*-mediated transformation of immature embryos derived from the cv. Golden Promise as described by Bieri *et al.* (2004). The presence of the transformed DNA fragments in the T_0 plants was checked by PCR assay with gene specific primer pairs (Tab. 2.6). T_1 progenies were bred through self-pollination of the T_0 plants. A total of 30 independent lines were generated for each of the two transgenes. 10 indepent lines for each transgene (1/S1-T6, 4/S1-T6, 7/S1-T6, 8/S1-T6, 16/S1-T6, 17/S1-T6, 19/S1-T6, 25/S1-T6, 31/S1-T6 and 32/S1-T6 for *Nbs1-Rdg2a* and 41/S1-T7, 42/S1-T7, 46/S1-T7, 54/S1-T7, 56/S1-T7, 57/S1-T7, 60/S1-T7, 62/S1-T7, 64/S1-T7 and 71/S1-T7 for *Nbs2-Rdg2a*), Thibaut, Golden Promise, Mirco, NIL3876 and the line 15/S1-T6 (with the empty vector) were infected with *P. graminea* isolates *Dg2* and *Dg5* using the 'sandwich' technique (Pecchioni *et al.*, 1996; Par. 2.3).

2.5.2 Analysis of T_1 progenies of transgenic plants

After infection with *Dg2*, DNA from leaves of 197 resistant and 10 susceptible transgenic plants for *Nbs1-Rdg2a* and 84 susceptible transgenic plants for *Nbs2-Rdg2a* was extracted by the Wizard Magnetic 96 DNA Plant System (Promega). The presence or the absence of the transgenes was tested by PCR with the same protocol described in Par. 2.4.3. PCR products were size fractionated on 1% agarose gel.

2.5.3 Expression analysis of the transgenes in T_1 progenies

To verify whether the transgenes are transcribed in resistant or susceptible plants, semi-quantitative RT-PCRs were performed. Total RNA from leaves of resistant and susceptible plants was extracted by the use of TRIZOL® Reagent (Life Technologies). RT-PCRs were conducted as described in Par. 2.4.4.1. The primer pairs were the same utilized to observe the presence or the absence of the genes in transformed genomes (Tab. 2.5). Moreover, to verify the presence of the fungus in infected tissues, specific primers for *P. graminea Ubiquitin* (FC555903 – primer forward: GACAGCACGTCTCATCTTCG, primer reverse: TCATATCCTCGTCCACGACA) and *P. graminea GTPase activator* (FC555890 – primer forward: CTCATAAGCCCGAGCACTTC, primer reverse: ATACCAAGGTACGGCTGCTG) were also used. PCR products were loaded on 1% agarose gel.

2.5.4 Southern blot analysis

Genomic DNA was extracted from leaves of the transgenic lines 8/S1-T6, 7/S1-T6, 4/S1-T6, 16/S1-T6, 25/S1-T6, 32/S1-T6 and from cvs. Thibaut and Golden Promise using the CTAB (*C*etyl *t*rimethyl *a*mmonium *b*romide) method (Doyle and Doyle, 1990).

8 µg of DNA were digested for 8 hours by 4 U/µg of EcoRI (Fermentas) and further 8 µg were digested by 4 U/µg of KpnI (Promega). The two sets of digested DNAs were loaded on 0.8% agarose gel, transferred to positively charge nylon filters using SSC 20X (Sambrook *et al.*, 1989) and fixed at 80°C for 2 hours.

A specific probe was obtained by PCR from the LRR region of *Nbs1-Rdg2a*, using primers D2_13 (GTTGCTACAGGTATCGGCATCACTAAGAGC) and Nbs1_10

(GCTGCAACCATCAATCATCAGATCTCGC)

(GTTGCTACAGGTATCGGCATCACTAAGAGCATTGTGTTGGCCCTCTTATTCAGTTGTCAT
TTCCAAGGCCATAAATGCAAAACATTTACGGTATCTTGACCTCTCTGGGTCAGACATTGT
TAGATTGCCAGATTCAATATGGGTGTTGTATAACCTGCAAACACTGAGGCTAATGGATT
GCCGGAAGTTGCGACAGTTACCAGAAGACATGGCAAGATTAAGAAAGCTCATCCATCTT
TACCTTTCTGGCTGTGAGAGTCTCAAAAGTATGTCTCCAAACTTTGGTCTGCTGAACAAC
CTTCACATATTAACAACATTTGTTGTGGGTTCCGGAGATGGCCTTGGAATAGAGCAGCTC
AAAGATTTGCAAAACCTTAGCAATAGGTTGGAAATATTGAATATGGACAAGATAAAGAG
TGGGGAGAATGCAAAAGAAGCCAATCTCAGTCAGAAGCAAAATCTAAGTGAGTTGTTGT
TCTCTTGGGGCCAAAAAATAGATGATGAGCCTACAGATGTGGAAGAAGTGCTTCAGGGC
TTAGAACCTCATAGTAATATCCAAAAACTGGAGATACGTGGATATCATGGCCTAGAAAT
ATCACAATGGATGAGAAAGCCTCAGATGTTTGACTGCTTGAGAGAACTCGAAATGTTTG
GCTGCCCAAAATGCAAGAGTATCCCTGTAATATGGTTCTCGGTCTCTCTAGAGATTTTGG
TCTTACAGAGCATGGATAACCTGACAACATTATGTAGTAACCTTGGTGTGGAAGCTGGA
GGAAGCATTACCCCTCTGCAACTTTTCCCAAATTTGAAGAAGTTGTGTTTGATTAAGTTA
CCAAGCCTGGAGATATGGGCAGAAAATAGTGTAGGAGAGCCTCGGATGTTTAGCAGTTT
GGAAAAACTCGAAATTTCCGACTGCCCAAGATGCAAGAGTATACCTGCAGTATGGTTTT
CGGTCTCTCTTGAGTTTTTGGTCTTACGGAAAATGGATAACCTGACAACATTATGTAATA
ACCTTGATGTGGAAGCTGGAGGATGCATTACCCCTATGCAGATTTTCCCAAGGTTGAAG
AAGATGAGGTTGATTGAGTTACCAAGCCTGGAGATGTGGGCAGAAAATAGTATGGGAG
AGCCTAGTTGTGATAACCTGGTAACATTCCCGATGCTTGAAGAGCTAGAGATCAAAAAT
TGCCCCAAGCTTGCAAGTATTCCAGCGATTCCCGTTGTCAGCGAGTTGAGAATAGTTGGA
GTTCACAGTACTGCAGTCGGTTCAGTTTTTATGAGCATCCGTTTGGGCTCCTGGCCATTTC
TCGTCAGGTTAAGTCTTGGGTCTCTAGAAGACATACCCATGTTGCCTCTAGACGCCCAGC
AAAACCAAAGTGAAAGACCTCTTGAAAAGCTTGAGAGTTTGACTCTGGAAGGGCCCAAC
AGCTTGATCAGAAGCTCTGGATTGTCCGGATCACAACTTATGGTTTGGAAATGTTTTCGG
TTCGTGCGAGATCTGATGATTGATGGTTGCAGC). 80 ng of the probe were labelled with α-^{32}P-dCTP using the DNA Polymerase Large (Klenow) Fragment (Promega). Marked probe was then purified on Sephadex columns (Sambrook *et al.*, 1989).

The filter was pre-hybridized over night at 65°C in 15 ml of Hybridization Buffer (0.5 M Sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA, 10 mg/ml denatured Harring sperm DNA). Hybridization was performed incubating over night at 65°C the filter in 15 ml of new Hybridization Buffer with the denatured α-^{32}P-dCTP labelled probe. The filter was washed to medium stringency (Sambrook e*t al.*, 1989) and subjected to autoradiography using the Biomax MS (Kodak) system.

2.6 *In situ* analyses

2.6.1 Sectioning of the embryos

Embryos of NIL3876 grown at 14, 22 and 26 dai in presence of *Pyrenophora graminea* isolate *Dg2* and, at the same time points, on sterile moist filter paper (control), were extracted from grains and immediately fixed by incubation in freshly prepared FAE (50% ethanol, 5% acetic acid and 3.7% formaldehyde in *P*hosphate *B*uffer *S*aline (PBS) (130 mM NaCl, 7 mM Na_2HPO_4 and 3 mM NaH_2PO_4)) with vacuum for 20 minutes and then were incubated in new FAE for 12 hours. The fixed material was placed in 70% ethanol and stored at 4°C.

After dehydratation using increasing concentration of ethanol, embryos were embedded in Paraplast Plus (Sigma Aldrich) and orientated to obtain longitudinal sections. The embedded embryos were stored at 4°C.

10 µm thick longitudinal sections, obtained using a microtome LEITZ 1512, were put on Polysine™ Microscope Slides (Biooptica) and store at 4°C.

Paraplast was removed from sections by the use of Histochoice Clearing Agent (Sigma Aldrich) and sections were rehydratated with decreasing concentration of ethanol in 0.85% NaCl.

2.6.2 *T*erminal deoxynucleotidil tranferase-mediated d*U*TP *N*ick and *L*abelling (TUNEL) and Autofluorescence assays

Sections of the embryos were permeabilized by treatment in 100 mM Tris/HCl, 50 mM EDTA, pH 8 with 20 µg/µl of recombinant Proteinase K PCR grade (Roche) for 45 min at 37°C.

After washing twice in PBS (Par. 2.6.1) for 1 min, sections were incubated with the TUNEL reaction mix (Label Solution with fluorescein-coniugated dUTP and Enzyme Solution with Terminal transferase) for 1 h and 30 min at 37°C, according to the *In Situ* Cell Death Detection Kit, Fluorescein (Roche).

Two negative controls were performed without Terminal transferase and two positive controls (one with an inoculated embryo and one with a control embryo) were carried out by incubation in 20 mM Tris/HCl, 2 mM $MgCl_2$, pH 8 with 5 U/ml

DNaseI (Sigma), for 3 min at 25°C before the treatment with the TUNEL reaction mix.

After the washing in PBS, samples were observed with an Olympus BX51 microscope fitted with the following configuration: excitation at 451-490 nm and emission at 491-540 nm for fluorescein and excitation at 335-380 nm and emission at >420 nm for autofluorescence. Images were recorded by an Olympus DP50 microscope digital camera system.

2.6.3 4',6-Diamidino-2-phelindole clorihydrate (DAPI) staining

To verify the presence of the nuclei in the cells that did not show a positive TUNEL signal, sections were incubated in 1% (w/v) 4',6-Diamidino-2-phelindole clorihydrate (DAPI) in PBS pH 7 (Par. 2.6.1) for 20 min at room temperature in the dark and were observed with an Olympus BX51 microscope fitted with excitation at 335-380 nm and emission at >420 nm. Images were recorded by an Olympus DP50 microscope digital camera system.

2.6.4 Calcofluor staining

To visualize *Pyrenophora graminea* in the infected tissues, sections were incubated in 0.01% Calcofluor (Sigma Aldrich) in PBS pH 7 (Par. 2.6.1) for 30 min at room temperature and were observed with an Olympus BX51 microscope fitted with excitation at 335-380 nm and emission at >420 nm. Images were recorded by an Olympus DP50 microscope digital camera system.

2.7 Analysis of the hypothetical *Rdg2a* locus in cultivar Morex

2.7.1 Screening by PCR-based molecular markers

Steuernagel *et al.* (2009) sequenced 91 barcoded, pooled, gene containing Morex BACs using the 454-GS-FLX sequencer and assembled the sequences under interative change of parameters using the Newbler software (Roche). In particular, they obtained, from BAC HVVMRXALLhA425O23_c2, a 26,223 bp contig that,

when compared to Thibaut *Rdg2a* locus by the use of Vector NTI 9 (Invitrogen), showed a hypothetical gene homolog to *Nbs1/Nbs2-Rdg2a*. The comparative analyses revealed also that some regions of Thibaut *Rdg2a* locus are quite conserved in Morex, even if in the last cultivar several deletions were observed with respect to the resistant haplotype. Based on these observations, to verify the assembling, PCR-based molecular markers were developed with several primers that annealed at sequences within or flanking the deleted regions (Tab. 2.7; Fig. 3.18(a); Par. 3.7, Results).

Primer combination	**Sequence**	**Expected amplicon dimensions**	
		Thibaut	**Morex**
CR1	TCTGAACGGGCGGGCTTATCTGAG	5360bp	1064bp
CR2	CAGGAGGAGAAGCTGGAGAACAAG		
NCR1	GAAGACGGCGCAGGAAGGATCGG	471bp	/
CR2	CAGGAGGAGAAGCTGGAGAACAAG		
CR3	GCTTACACAGTGCCAATGCTAAGC	14872bp	1179bp
CR4	ATGGGCAATACCTGCACCTTCTTC		
CR3	GCTTACACAGTGCCAATGCTAAGC	1183bp	/
NCR2	TGATGGGCAGCACCTGCACCCTCCG		
Nbs2_30	TGAGGGTAGGCACACTGCACAC	1927bp	/
CR5	AGCTTATGGGTTCCAAGGGACTGC		

Tab. 2.7 Primer combinations used for the PCR-based molecular markers screening of Morex hypothetical *Rdg2a* locus and designed on the base of Thibaut sequence. The dimensions of the corrispective PCR amplicons in the two genotypes are also reported (Fig. 3.18(a); Par. 3.7, Results).

Furthermore, primers specific for Morex sequence were utilized (Tab. 2.8).

Primer combination	**Sequence**
Morex5fw	TCACCGGGCTTACACAGTGC
Morex7rev	AAGTCGTCGAGCACGTTGTCAGC
Morex9fw	TTCTCTGAGGGAATTATGGATTTGG
Morex11rev	TGATGATTACTTGTGGACAACAG
Morex13fw	AAACTTAGAATCCGGACAGGC
Morex18rev	ATGTATGATTGCACTCTTTTCCC

Tab. 2.8 Primer combinations used for the PCR-based molecular markers screening of Morex hypothetical *Rdg2a* locus and specific for Morex sequence.

PCRs were performed on Thibaut genomic DNA and on the Morex BAC 146G20, belonging to the Morex BAC library utilized for the *Rdg2a* positional cloning (Par. 1.3.1, Introduction). This BAC, on the basis of PCR and sequence analyses, demonstrated to overlap to the BAC 425O23 processed by Steuernagel e*t al.* (2009). PCR conditions were the same described in Par. 2.4.2 with the exclusion of the

annealing time that was of 2' in these last experiments. PCR products were loaded on a 1% agarose gel.

Finally, to determine whether a homolog gene of Thibaut *Nbs3-Rdg2a* is present in the Morex *Rdg2a* locus, several PCRs, using primer combinations specific for this gene (Tab. 2.9), were performed as described in Par. 2.4.2 (3' annealing) with both Thibaut genomic DNA (control) and Morex BAC 146G20.

Primer combination	Sequence
Nbs3_47	GCAGCCTTGACGCGCGAGAGACCAT
Nbs3_12	CTTCTGTTCTTGCTGGTCCAACAGTTT
Nbs3_46	CTTCTCTCCATTTTCCCAACAACCGC
Nbs3_11	TCAGGCATGTCACATCTTCCACTTAC
Nbs3_33	GCTGAGGAGATGAGCGAGAAGAAGTC
Nbs3_11	TCAGGCATGTCACATCTTCCACTTAC
Nbs3_36	AAGAGAGAACAATGGATTTAACACGGAA
Nbs3_27	CGGGCTATCAGACATTTTGAGTTCTC
Nbs3_29	AGAGATGTGGAAGAAGTGCTTCAGTGCT
Nbs3_49	CTGATCAAGCCGTTGAGCCCTTT

Tab. 2.9 Primer combinations used to verify whether Morex carries a homolog gene of Thibaut *Nbs3-Rdg2a*.

Amplified PCR products (Nbs3_29+Nbs3_49 and Nbs3_46+Nbs3_11) from Morex were purified from 1% agarose gel by the Wizard® SV Gel and PCR Clean-Up System (Promega) and directly sequenced using 2.5 ng each 100 bp of DNA and the same primers utilized for PCR reactions (Tab. 2.9). Sequencing outputs were analyzed by the Sequencing Analysis Software v5.2 with KB Basecaller Software v1.2 (Applied Biosystem, Foster City, CA USA) and were compared to Thibaut *Nbs1-Rdg2a*, *Nbs2-Rdg2a*, *Nbs3-Rdg2a* and to Morex contig by the use of Vector NTI 9 software (Invitrogen, Carlsbad, CA USA).

2.7.2 Sequencing of Morex BAC146G20

The region comprised between the Morex 26,223 bp-long contig and the amplicon obtained by the primer combination morex13fw+Nbs3_11 was sequenced by primer walking on the amplicons and directly on the Morex BAC using 1 µg of BAC 146G20 as template. The protocol is described in Par. 2.4.2. Primers used for sequencing are reported in Tab. 2.10.

Primer	Sequence
Morexwalkingfw1	CACGATGCAGAGAAGGTGGG
Morexwalkingfw2	TGGGGAGTATTTCCACTTGCTC
Morexwalkingrev1	CGTCAAGGCTGCTTGGTTTG
Morexwalkingrev2	TGCAGATCTCTCACGCGTCAAGG
Morexwalkingfwbac5	TGACAAAGGGGTCCAGCAACA
Morexwalkingfwbac6	ATTTCCAGTTGAAGATGTGGCACT
Morexwalkingrev5	TGCAAGGAAGCATTCGCTCA
Morexwalkingrev6	CCACAATAATAAACCAAGCC
Morexwalkingfwbac7	GCCTGTGGTGTCCCGCCAA
Morexwalkingfwbac8	AGCACGTTAGGCTACGGCTCA

Tab. 2.10 Primer used for the primer walking on Morex BAC 146G20.

2.7.3 Expression analysis in cultivar Morex

A two-step RT-PCR was performed to analyze the expression of the *Rdg2a* homolog gene in cv. Morex. RNA was extracted by the use of TRIZOL® from barley embryos grown at 7, 14 and 22 dai with *Pyrenophora graminea* isolate *Dg2* and at the same time points after growth on sterile moist filter paper (control). RNA was also extracted from leaves of 14 days old seedlings infected with the fungus and from control leaves. RT-PCRs were conducted as described in Par. 2.4.4.1. Primers used were specific for the Morex gene (nbs1-1-mo: GGTTCCTGGCCATTTCTTGCTGAG and nbs1-2-mo: CTGCATCGTGTTCCCAACTCCGG).

To complete the expression analysis, a quantitative RT-PCR was performed using the same RNA utilized for the RT-PCRs and following the protocol described in Par. 2.4.4.2. Primers, specific for the Morex gene, were projected by Primer Express software (Applied Biosystem) and were:

rtmorexfw5 CAAATGTCAAAGGCTGAATTCG

rtmorexrev5 CAAAGTGCGGAGGTATGTTCTG

2.8 Analysis of different barley varieties

2.8.1 PCR-based molecular markers analysis of different barley varieties

Different barley cultivars (Rika, Bulbul, Triumph, Imber, Optic, Ansis, Gitane, Bonus, Ketos, Grete, Franka, Marado and Federal) were tested for their susceptibility/resistance to *P. graminea* isolates *Dg2* or *Dg5* by the use of the

sandwich technique (Pecchioni *et al.*, 1996; Par. 2.3). Cvs. Thibaut, Mirco and Golden Promise were also tested as controls. An analysis of the haplotype (Thibaut, Morex and other un-identified haplotypes) was performed throught a PCR-based molecular markers screening in the above mentioned cultivars and in other varieties already characterized for their response to leaf stripe infection with isolates *Dg2* and *Dg5* (Rebelle, Onice, Proctor, Alf, Diadem, Haruna Nijo, Galleon, Jaidor, Nudinka, Passport and Acuario) (Arru *et al.*, 2003; Par. 2.2). The Wizard Magnetic 96 DNA Plant System (Promega) was utilized for the extraction of genomic DNA from the leaves of these cultivars and from Thibaut and Morex leaves (controls). PCRs were performed as described in Par. 2.7.1, using the same primer combinations utilized to verify the assembling of the Morex 26,223 bp-long contig (Tab. 2.7).

2.8.2 Sequencing of *Rdg2a* in cultivars Rebelle, Haruna Nijo and Galleon

Rdg2a alleles were amplified by PCR using the genomic DNA of cvs. Rebelle, Haruna Nijo and Galleon. *Nbs1*-specific primers obtained from Thibaut *Nbs1-Rdg2a* genomic sequence (Tab. 2.11) and Go Taq® DNA Polymerase (Promega) were utilized following the protocol described in Par. 2.4.2.

Fragment	Primer combination	Sequence
Fragment I	Nbs1_17	CACCGCATCATGAAGAGAACTGATACAGGA
	D2_19	CCTTGCCGGCCACGCCGCGCACTAG
Fragment II	Nbs1_14	TACTTGGTTTGGAGCTAGGAGACG
	Nbs1_10	GCTGCAACCATCAATCATCAGATCTCGC
Fragment III	D2_10	GTGTAGGAGAGCCTCGGATGTTT
	Nbs1_19	GGTACCATCGATTCATGACGTTAGCAT

Tab. 2.11 Primers used to amplify subsequent fragments of *Rdg2a* in cvs. Rebelle, Haruna Nijo and Galleon.

Primers used to sequence the fragments in the three cultivars are listed in Tab. 2.12.

Barley variety	Fragment	Primer	Sequence
Rebelle	I	Nbs1_1	CTTCACCGCGCTTACACAGTGCCA
		D2_12	ATTGAGAGGCCGCCAGTAGGTACC
		D2_1	CGCCAGTAGGTACCTACCAGTCAATAT
	II	Nbs1_9	ATTGGTCACATGTCGAAGCAAGCAAGTCG
		Nbs1_4	CAGGCGACAGGTGTTTGTAGCTTA
		Nbs1_21	GGCGTAACGGTGCACATTATC

		Nbs1_20	CACCCTGTTCTTGTACATGCTGCAGCTTCC
		D2_6	GACGTGCTTTGGAAGATAACAAGG
		D2_8s2	AGAAATATCACAATGGATGAGAA
		Nbs1_7	CCTCGGATGTTTAGCAGTTTGGA
		Nbs1_12	TCATCAGATCTCGCACGAACCGA
		UTR_R1	TTGCTTGCTTCGACATGTGACC
		UTR_R2	GATGTTGCCGCTCCTCCCTACG
		Nbs1_11	GTAACATCGGGGATAAAGATGGAGGC
		D2_16s3	GCACATACTGAAGTTAAGCT
		Nbs1_34	AAATCCTTGTGTGATCCTGAAGGAA
		Nbs1_2	GGCCAACACAATGCTCTTAGTGATGCCGAT
		Nbs1_26	GCTAAACATCCGAGGCTCTCCTACACTA
		D2_17	TCGCAACTTCCGGCAATCCATTAG
		D2_8s1	AAACCTTAGCAATAGGTTGGA
		Nbs1_25	GATGAGCCTACAGATGTGGAAGAAGTGC
	III	Nbs1_15	CAGAACTGCCGCAGTGTAGTAGC
		D2_14	TATGCCGATTCACTTTGGGATGCCTATTC
		D2_15	GGAACAGAGGAGAGCAAGTGGAAGTAC
		D2_2	CACCTTCTCTGCATCGTCTTTGC
		Nbs1_12	TCATCAGATCTCGCACGAACCGA
		D2_13	GTTGCTACAGGTATCGGCATCACTAAGAGC
		D2_8s1	AAACCTTAGCAATAGGTTGGA
		D2_8s2	AGAAATATCACAATGGATGAGAA
		Nbs1_25	GATGAGCCTACAGATGTGGAAGAAGTGC
		Nbs1_5	TAGTGTAGGAGAGCCTCGGATGTT
		Nbs1_7	CCTCGGATGTTTAGCAGTTTGGA
		Nbs1_35	GGCAGTCGGAAATTTCGAGTTTTTCC
		Nbs1_36	GGAAAAACTCGAAATTTCCGACTGCC
		Nbs1_10	GCTGCAACCATCAATCATCAGATCTCGC
Haruna Nijo	I	D2_11	CCTTACCAACGCCCAAATTTGTCG
		D2_1	CGCCAGTAGGTACCTACCAGTCAATAT
		D2_18	TTTCCCATACCAAGCAGAGCCTTCGA
	II	Nbs1_4	CAGGCGACAGGTGTTTGTAGCTTA
		Nbs1_11	GTAACATCGGGGATAAAGATGGAGGC
		Nbs1_21	GGCGTAACGGTGCACATTATC
		D2_13	GTTGCTACAGGTATCGGCATCACTAAGAGC
		D2_16s3	GCACATACTGAAGTTAAGCT
		Nbs1_34	AAATCCTTGTGTGATCCTGAAGGAA
		Nbs1_2	GGCCAACACAATGCTCTTAGTGATGCCGAT
		D2_5	GGAAATGACAACTGAATAAGAGGGCC
		D2_17	TCGCAACTTCCGGCAATCCATTAG
		UTR_R2	GATGTTGCCGCTCCTCCCTACG
		Nbs1_20	CACCCTGTTCTTGTACATGCTGCAGCTTCC
		D2_8s2	AGAAATATCACAATGGATGAGAA
		Cos179	TTGGGCAGCCAAACATTTCGA
		Nbs1_5	TAGTGTAGGAGAGCCTCGGATGTT
		D2_10	GTGTAGGAGAGCCTCGGATGTTT
		Nbs1_7	CCTCGGATGTTTAGCAGTTTGGA
		Nbs1_25	GATGAGCCTACAGATGTGGAAGAAGTGC
		Nbs1_26	GCTAAACATCCGAGGCTCTCCTACACTA
		Nbs1_35	GGCAGTCGGAAATTTCGAGTTTTTCC
		Nbs1_12	TCATCAGATCTCGCACGAACCGA
		D2_6	GACGTGCTTTGGAAGATAACAAGG
		D2_16	CTGTTCTTGTACATGCTGCAGCTTCC
		D2_19	CCTTGCCGGCCACGCCGCGCACTAG

	III	Nbs1_15	CAGAACTGCCGCAGTGTAGTAGC
		Nbs1_16	AGCTGAGGAGTCTCTATGTGAGCG
		D2_9	CCATTGGTTATCACCTAATTTGTAT
		D2_7	TTTGTCAGGTAAGGAGACTCACGC
		D2_14	TATGCCGATTCACTTTGGGATGCCTATTC
		D2_15	GGAACAGAGGAGAGCAAGTGGAAGTAC
Galleon	I	Nbs1_1	CTTCACCGCGCTTACACAGTGCCA
		D2_6	GACGTGCTTTGGAAGATAACAAGG
		D2_11	CCTTACCAACGCCCAAATTTGTCG
		D2_3	TTTCTTAGCTGTGCAAAACATCC
		Nbs1_14	TACTTGGTTTGGAGCTAGGAGACG
	II	D2_6	GACGTGCTTTGGAAGATAACAAGG
		D2_16	CTGTTCTTGTACATGCTGCAGCTTCC
		D2_19	CCTTGCCGGCCACGCCGCGCACTAG
		UTR_R2	GATGTTGCCGCTCCTCCCTACG
		Nbs1_20	CACCCTGTTCTTGTACATGCTGCAGCTTCC
		Nbs1_23	CTTATCATCTTCCCTTCCAA
		Nbs1_31	CCGCTGATCCTGCTGGTCCAGCAGCAA
		D2_16s2	CGAACTATTGCAAAAGAAAC
		Nbs1_9	ATTGGTCACATGTCGAAGCAAGCAAGTCGC
		UTR_R1	TTGCTTGCTTCGACATGTGACC
		Nbs1_21	GGCGTAACGGTGCACATTATC
		Nbs1_22	CGGTGCACATTATCGAGGCG
		Nbs1_11	GTAACATCGGGGATAAAGATGGAGGC
		Nbs1_4	CAGGCGACAGGTGTTTGTAGCTTA
		Nbs1_34	AAATCCTTGTGTGATCCTGAAGGAA
		Nbs1_2	GGCCAACACAATGCTCTTAGTGATGCCGAT
		D2_13	GTTGCTACAGGTATCGGCATCACTAAGAGC
		D2_17	TCGCAACTTCCGGCAATCCATTAG
		Nbs1_25	GATGAGCCTACAGATGTGGAAGAAGTGC
		D2_8s2	AGAAATATCACAATGGATGAGAA
		Cos179	TTGGGCAGCCAAACATTTCGA
		D2_10	GTGTAGGAGAGCCTCGGATGTTT
		Nbs1_26	GCTAAACATCCGAGGCTCTCCTACACTA
		Nbs1_7	CCTCGGATGTTTAGCAGTTTGGA
		Nbs1_36	GGAAAAACTCGAAATTTCCGACTGCC
		Nbs1_35	GGCAGTCGGAAATTTCGAGTTTTTCC
		Nbs1_12	TCATCAGATCTCGCACGAACCGA
	III	Nbs1_15	GATGAGCCTACAGATGTGGAAGAAGTGC
		Nbs1_16	AGCTGAGGAGTCTCTATGTGAGCG

Tab. 2.12 Primers used for the sequencing of *Rdg2a* in cvs. Rebelle, Haruna Nijo and Galleon.

2.8.3 Expression analysis of *Rdg2a* in cultivars Rebelle, Haruna Nijo and Galleon

Total RNA was extracted from leaves of cvs. Rebelle, Haruna Nijo and Galleon by the use of TRIZOL® Reagent (Life Technologies) and was treated by DNaseI (Ambion). cDNAs were synthesized, as described in Par. 2.4.4.1, and RT-PCRs were performed with the same primer combinations utilized for Thibaut *Nbs1-Rdg2a* (Tab. 2.6).

3. Results

3.1 Sequencing of *Nbs3-Rdg2a* cDNA from NIL3876

A previous RiceGAAS (http://ricegaas.dna.affrc.go.jp/, Sakata *et al*., 2002) analysis of the *Nbs3-Rgd2a* genomic sequence in the resistant cv. Thibaut (Bulgarelli *et al.*, 2010) predicted that this gene consists of four exons and three introns of 167 bp, 792 bp and 56 bp, positioned 2,075 bp, 3,442 bp and 4,706 bp downstream the start codon, respectively. To investigate whether these introns were subjected to splicing or to alternative splicing during infection, a RACE (*R*apid *A*mplification of *c*DNA *E*nds) analysis and semiquantitative RT-PCRs were performed to obtain full length cDNA of *Nbs3-Rdg2a*. Poly(A)RNA extracted from NIL3876 (Par. 2.2; Marials and methods) embryos inoculated for 7 days with leaf stripe isolate *Dg2* and from 7 days old embryos grown in absence of the fungus (control) and primer pairs generating overlapping fragments along the entire coding sequence of *Nbs3-Rdg2a* were utilized. Amplification products were then cloned into the Zero Blunt TOPO PCR vector (Invitrogen) for sequencing. Amplicons that did not show any difference with respect to the genomic sequence were processed and assembled by Vector NTI10 software (Invitrogen, Carlsbad, CA USA).

None of the predicted introns was found to be spliced in both control and inoculated embryo tissues, leading to the introduction of stop codons in the mRNA sequence. This analysis revealed instead the presence of three introns: the first is 305 bp long, located within the 5'UTR, 332 bp upstream the start codon and subjected to splicing; the second is a 44 bp-long intron spliced out in only a third (4/12) of the RACE clones analyzed and located at 105 bp after the ATG; the third is positionated within the 3'UTR, at 6,416 bp after the ATG, just after the stop codon; it is 70 bp long and is subjected to splicing. In particular, splicing of the 44 bp intron results in the generation of a stop codon after the splicing site, while retaining of this intron, due to alternative splicing pattern, generates a stop codon in the first not spliced predicted intron at position 2,388 bp with respect to the start codon (Fig. 3.1), thus producing a truncated protein with only three LRR units.

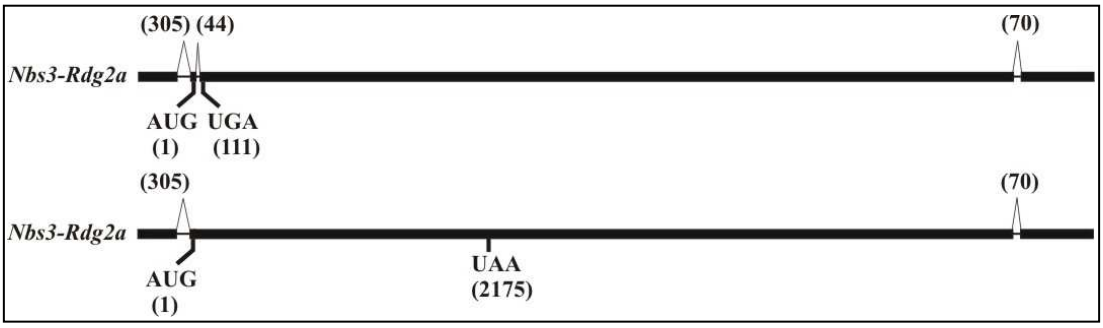


Fig. 3.1 Schematic diagram of the two transcript types resulting from alternative splicing pattern of Thibaut *Nbs3-Rdg2a*. Solid bars indicate the transcribed regions, lines angled upwards indicate the positions of spliced introns and lines under the solid bar indicate the positions of not spliced predicted introns. Sizes of the real and predicted introns, the positions of ATG, TGA and stop codon TAA are indicated in brackets (Bulgarelli *et al.*, 2010).

Based on these results, *Nbs3-Rdg2a* was excluded form the *Rdg2a* candidates because it probably encodes severely truncated non-functional proteins.

3.2 Sequencing of *Nbs1-rdg2a* and *Nbs2-rdg2a* in cultivar Mirco

To highlight differences between Thibaut and Mirco alleles for the *Nbs1-Rdg2a* and *Nbs2-Rdg2a* genes, sequencing of Mirco alleles was carried out. Overlapping fragments belonging to the coding region, 5' and 3'UTR and putative upstream regulatory regions of the two genes were amplified, directly sequenced and assembled using Vector NTI10 software (Invitrogen, Carlsbad, CA USA).

Mirco *Nbs1-rdg2a* coding region (accession number HM124453) showed 85% of sequence identity with respect to Thibaut *Nbs1-Rdg2a* (accession number HM124452). Several frameshift sites resulting in stop codons together with nonsense mutations were identified. Moreover, the putative promoter showed different rearrangements and, in particular, two main insertions of 436 bp and 854 bp, respectively (Fig. 3.2A). The first insertion is positioned 459 bp upstream the start codon and 96 bp upstream the transcription start site, after a putative TATA-box element (this last is located 494 bp upstream the ATG); while the second insertion is within the putative transcribed region, 65 bp upstream the start codon. No homologies with repeat sequences were identified in the two insertions, but in the longer, flanking sequences represented by two inverted repeats of 138 bp each were found

(**GAGAAAATTGGATATTTGCCACTTTAAACATCTGGTTTCGCAGAACTGCCACTCTC**
AAGATGGGCTTCGCAAAAATGCCACCTGGCTCGTGGACACCTTGATTACCATGCCA
TTTTCCCTCTTTTATTGATTTTCCTTTTGCTTTTCCATTTACGTGCACCTAAAAGGACTA
GACTGCCCCTGTTCGTGCCGAAGCCCCTTCTTGCCTGCTCGTCACTCTCACCTTCTCACCG
CCGCAACTCGCCCTTGCATGCTCGCCGTCACAGCTCACCTCTGCTCCGCTCGCCCACACT
TGCGCTCGCTGCTGCAGCTCGTCAGGCGACGTGCTGAAGGAGTGGCGGAGGCAGGCACG
AACACAGGCATCAGTGATGCAACAACGGCAATAGCTGGACCCTGTTGTACGTTCTTGGA
GGAAGGAGAACAATGTACGTGCAGGTAGTGAAGATAGATTTTGGCAGCGTCGAGGCGG
TCGACGGCGATGGCGCCCGGGGAATCGGCAGCGCAGCGGAGTTCAGGAGTTTACAGTG
GCCACGTACAGGAGATCAGATTCGGCGATGTCTAGGCGATCCTAGGCAATTAGTGACTG
CGCGGTGTTCTTTGTTGTTGTTGTATTGGCTAGCTACCCGTGCGTCTGTACAATTAGATCA
GGTACGTGTACACATCCAAGGGTAGTGTGGTCTATTCAGGTGCATCTAAATGCAAAAGC
AAGGAAAATCAATAAAAGAGGAAAAATGGCATGGTAATCAAGGTGTCCACAAGCCA
GGTGGCATTTTTGTGAAGCCCATCTTGAGAGTGGCATTTCTGCGAAGCCAGATGTT
TAAAATGGCAAATATCCAATTTTCTC (inverted repeats are in bold)).

Mirco *Nbs2-rdg2a* coding region (accession number HM124454) showed 94% of sequence identity with the *Nbs2-Rdg2a* allele (accession number HM124452) and no stop codons were identified within the coding sequence. Interestingly, sequencing of the putative promoter region revealed a 347 bp deletion in the Mirco haplotype with respect to Thibaut at 145 bp upstream the transcription start site (Fig. 3.2B). BLAST search of the deleted sequence in the *Triticeae Rep*eat Sequence (TREP) database (http://wheat.pw.usda.gov/ITMI/Repeats/) revealed 88% of sequence identity to the *Stowaway* type of *M*iniature *I*nverted *T*ransposable *E*lement (MITE) sequence. MITE elements are often associated with genes in crop species (Choulet *et al.*, 2010; Wicket *et al.*, 2006; Sabot *et al.*, 2005; Wesser *et al.*. 1995). Two direct repeats of 41 bp each (**CATTTTCCCAACAACCGCACAAACCCAAACCAAGCAGCCTT**CACACGAGAGAGAT**C**
ATTTTCCCAACAACCGCACAAACCCAAACCAAGCAGCCTT; inverted repeats are in bold) were additionally found in Mirco, while only one of these repeats is maintained in Thibaut and terminates just upstream the transcription start site at 470 bp from the ATG (Fig. 3.2B).

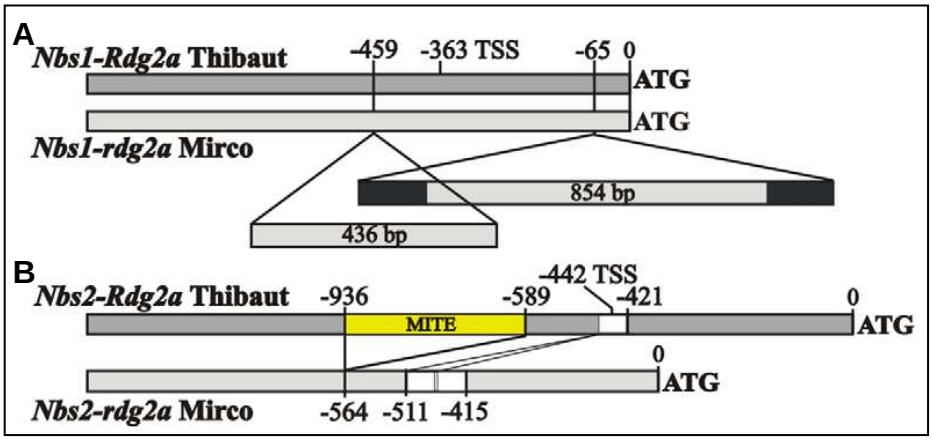


Fig. 3.2 Structural differences between Thibaut and Mirco alleles of *Nbs1-Rdg2a* and *Nbs2-Rdg2a* in the putative promoter regions. Positions of insertion/deletions relative to the start codon are shown. Filled sections indicate inverted repeats present in an insertion in the Mirco *Nbs1-rdg2a* gene (**A**). The *Nbs2-Rdg2a* allele comparison illustrates variation for a MITE insertion and a 41 bp direct repeat (open sections) (**B**). The *T*ranscription *S*tart *S*ites (TSS) for the two genes in the resistant genotype are indicated (Bulgarelli *et al.*, 2010).

To verify whether the Mirco sequences are true alleles, primers were chosen overlapping flanking regions of the insertions, for *Nbs1-rdg2a*, and the deletion, for *Nbs2-rdg2a*, and the obtained markers were used to verify co-segregation of the insertion/deletion polymorphisms with the *Rdg2a* locus in selected rare recombinants identified from a high resolution genetic mapping population (2,800 F_1 gametes). The In/Del markers (Nbs1_14+Nbs1_19 and Nbs2_6+Nbs2_29; Fig. 3.3A) co-segregated with the *Rdg2a* locus (Fig. 3.3B and C), demonstrating that the Mirco sequences represent true alleles of *Nbs1-Rdg2a* and *Nbs2-Rdg2a*.

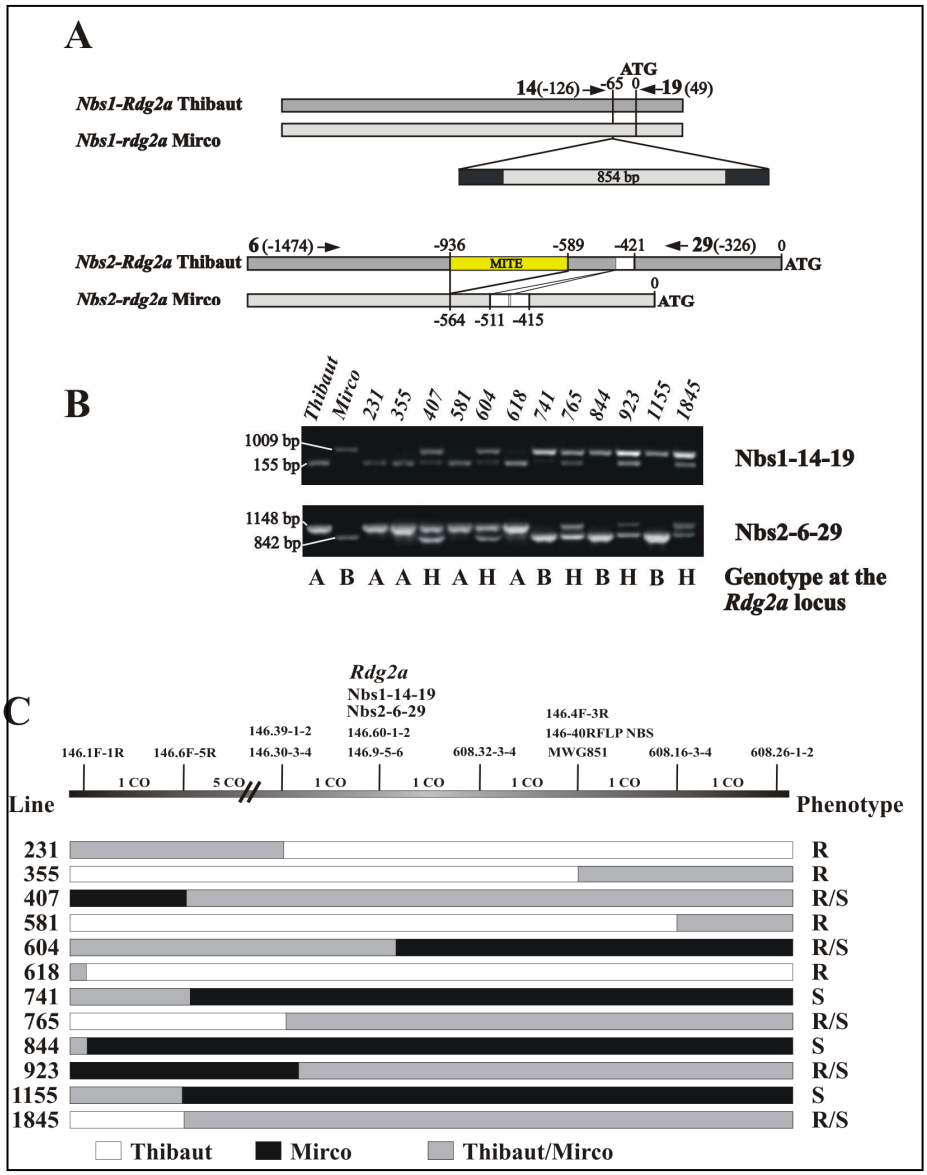
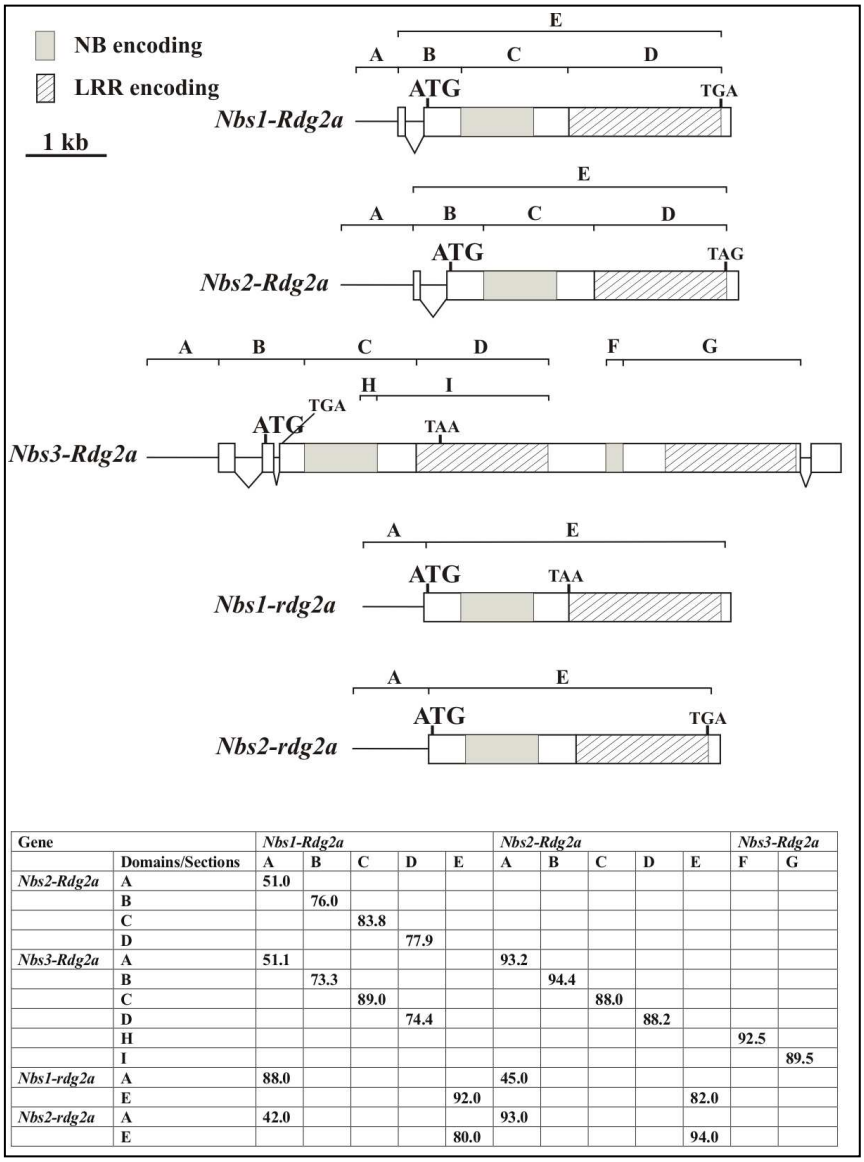


Fig. 3.3 Demonstration that the sequenced Mirco *Nbs1-rdg2a* and *Nbs2-rdg2a* genes represent alleles of the respective Thibaut genes. Markers Nbs1_14+Nbs1_19 and Nbs2_6+Nbs2_29, developed using insertion/deletion polymorphisms in the putative regulatory regions (**A**), cosegregated with the *Rdg2a* locus in 12 rare recombinants for the *Rdg2a* region that had been identified in the high resolution mapping population (**B**). Recombination points are illustrated in **C** (Bulgarelli *et al.*, 2010).

Fig. 3.4 summarizes the DNA sequence homologies between paralogs and alleles at the *Rdg2a* locus in cvs. Thibaut and Mirco.



Gene		*Nbs1-Rdg2a*					*Nbs2-Rdg2a*					*Nbs3-Rdg2a*	
	Domains/Sections	A	B	C	D	E	A	B	C	D	E	F	G
Nbs2-Rdg2a	A	51.0											
	B		76.0										
	C			83.8									
	D				77.9								
Nbs3-Rdg2a	A	51.1					93.2						
	B		73.3					94.4					
	C			89.0					88.0				
	D				74.4					88.2			
	H											92.5	
	I												89.5
Nbs1-rdg2a	A	88.0					45.0						
	E					92.0					82.0		
Nbs2-rdg2a	A	42.0					93.0						
	E					80.0					94.0		

Fig. 3.4 DNA sequence homologies between paralogs and alleles at the *Rdg2a* leaf stripe resistance locus. Diagrams above define the domains compared. Percent identities were determined once major insertions/deletion differences had been removed (Bulgarelli *et al.*, 2010).

3.3 Expression analysis of the *Rdg2a* candidates

To examine the genotype- and tissue-dependent expression patterns of the *Nbs1-Rdg2a* and *Nbs2-Rdg2a* genes, gene-specific semiquantitative RT-PCRs were carried out using two different primer combinations for each candidate. cDNA were synthesized starting from DNaseI treated RNA isolated from NIL3876 and Mirco embryos grown in the presence of *P. graminea* isolate *Dg2* at 7 and 14 *d*ays *a*fter *i*noculation (dai) and in control conditions (non-inoculated) at the same time-points,

as well as from non-inoculated NIL3876 and Mirco leaves. Since *Nbs-LRR* genes are quite conserved, the two different primer pairs, specific for the different genes in the two genotypes, were designed in the more specific and less conserved LRR encoding domain (Fig. 3.4(a)). Barley *Actin* gene was used as the reference gene. After amplification the identity of the amplicons was checked by sequencing.

The two genes were found to be transcribed only in the embryos tissues and leaves of the resistant cultivar (Fig. 3.4(b)).

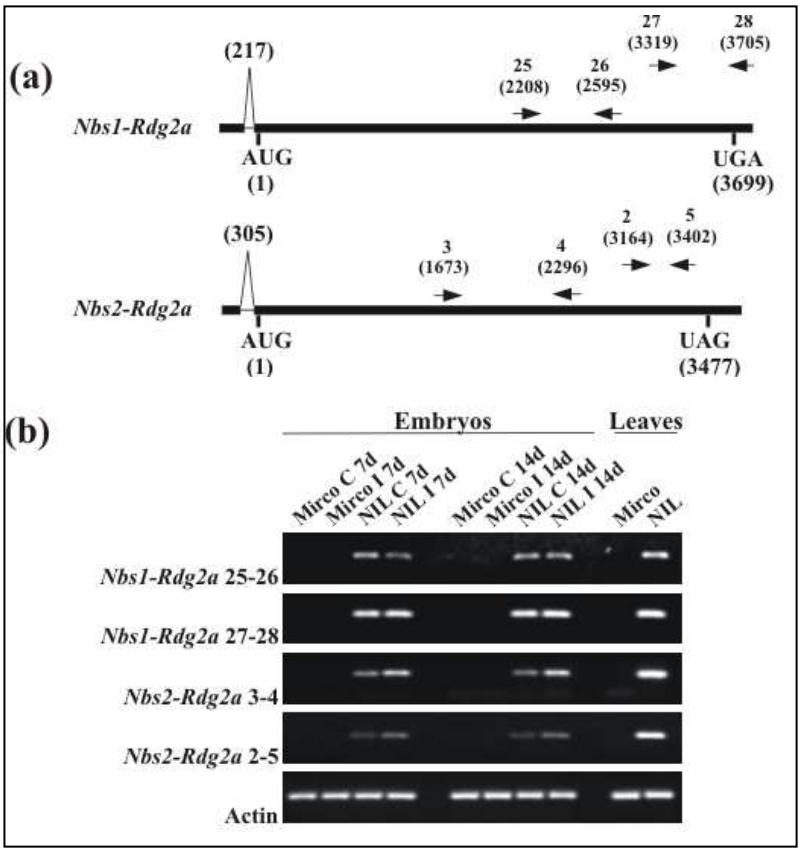


Fig. 3.4 (a) Schematic representation of the *Nbs1-Rdg2a* and *Nbs2-Rdg2a* transcripts. Solid bars indicate the transcribed regions and lines angled upwards indicate the positions of introns, whose sizes are shown. Open reading frames are indicated by start and stop codons. Arrows and numbers in branckets represent the positions of the primers used for semiquantitative and quantitative RT-PCRs, referring to Thibaut haplotype. **(b)** RT-PCR analysis of the *Rdg2a* candidates in cv. Mirco and NIL3876 embryos under control conditions and after inoculation with *P. graminea* isolate *Dg2* at two time-points (7 and 14 dai) and in leave tissues. Barley *Actin* gene was used as an internal control. (Bulgarelli *et al.*, 2010)

Furthermore, quantitative RT-PCRs in control and inoculated embryos at five time-points (7, 14, 18, 22 and 26 dai) were conducted. One primer combination for each gene was chosen for the analysis (Nbs1_27+Nbs1_28 for *Nbs1-Rdg2a* and Nbs2_2+Nbs2_5 for *Nbs2-Rdg2a*) (Fig. 3.4(a)); these primer combinations were

located close to the putative poly-A site of the mRNAs and generated amplicon lengths of about 100 bp. The analysis was carried out using plant materials of two independent biological replicates and eight technical replicates for each of them. The results were expressed as relative transcription of each gene, normalized to the expression of barley *Actin*, compared to the expression of the same gene in unchallenged conditions.

Following leaf stripe inoculation, the expression of *Nbs2-Rdg2a* increased 2.6-3.4 times from 7 to 18 dai and then declined toward the level of uninoculated embryos by 22 dai. In contrast, no substantial changes in gene expression as a response to leaf stripe infection were observed for *Nbs1-Rdg2a* (Fig. 3.5).

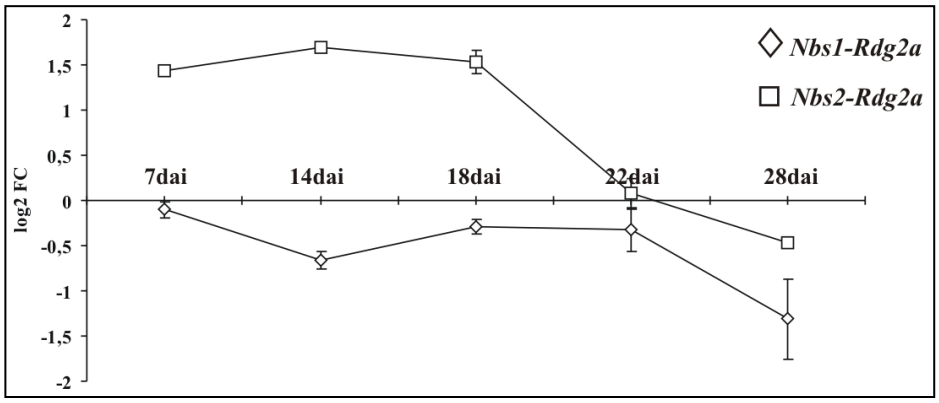


Fig. 3.5 Quantitative RT-PCR at 7, 14, 18, 22 and 26 dai for the two candidates in NIL3876 embryos. Values are expressed as log2 *F*old *C*hanges (FC) of transcript levels in the inoculated samples with respect to the transcript levels in uninoculated barley embryos. Error bars represent SD (*S*tandard *D*eviation) across all RT-PCR replicates (eight for each of two independent inoculations) (Bulgarelli *et al*., 2010).

A comparison between the expression levels of the two genes was also performed and it was verified that the *Nbs2-Rdg2a* transcript was found to be 2 to 16 times less abundant than that of *Nbs1-Rdg2a*, depending on the time-point and inoculation treatment (data not shown).

3.4 Identification of the *Rdg2a* gene

Genomic clones of the coding sequences under the control of their native promoters and terminators were generated for the two *Rdg2a* candidates (*Nbs1-Rdg2a* and *Nbs2-Rdg2a*) and used to transform the leaf stripe susceptible barley cv. Golden Promise. A total of 30 independent lines were generated for each transgene and T_1

seeds, derived from T_0 plants examinated by PCR for the presence of the transgene (Bulgarelli *et al.*, 2010), were tested for resistance against the isolates *Dg2* and *Dg5* in triplicate experiments, using the sandwich technique (Pecchioni *et al.*, 1996).

Transgenic lines for the *Nbs1-Rdg2a* gene were segregating for resistance to isolate *Dg2* and were fully susceptible to isolate *Dg5*, towards which the *Rdg2a* gene is ineffective (Tab. 3.1). Moreover, to verify whether the resistance co-segregates with the Thibaut *Nbs1-Rdg2a* allele, PCR analyses were performed on genomic DNA of resistant and susceptible plants belonging to the transgenic line 16/S1-T6. Primer pairs generating amplicons of different sizes in the resistant cv. Thibaut and in the susceptible cv. Golden Promise genomic backgrounds were used. All the resistant transgenic plants showed the presence of the Thibaut allele (in addition to the Golden Promise allele), while susceptible plants amplified the Golden Promise allele only (Fig. 3.6(a)). Moreover, the expression of Thibaut *Nbs1-Rdg2a* for resistant plants was verified by RT-PCR using gene-specific primers and cDNAs derived from DNaseI-treated RNAs extracted from the leaves of these plants (Fig. 3.6(a)). The same analysis was conducted on T_1 lines transformed with *Nbs2-Rdg2a* and all of them were fully susceptible to both the leaf stripe isolates (Tab. 3.1). The overall escape rate of 5% among the null segregants was similar to the value observed in the susceptible control varieties (data not shown). RT-PCRs showed that this gene is also transcribed in these plants (Fig. 3.6(b)).

These results led to the conclusion that *Nbs1-Rdg2a* is the *Rdg2a* gene as it confers the same resistance specificity.

		Isolate Dg2		Isolate Dg5	
Constructs/barley cvs.	**Lines[a]**	**No. plants[a]**	**No. res. plants[b]**	**No. plants**	**No. res. plants**
Nbs1-Rdg2a	1/S1-T6	19	19	15	0
	4/S1-T6	21	21	13	0
	7/S1-T6	24	24	11	0
	8/S1-T6	23	22	5	0
	16/S1-T6	19	19	12	0
	17/S1-T6	15	14	8	0
	19/S1-T6	7	7	9	0
	25/S1-T6	19	19	12	0
	31/S1-T6	13	13	5	0
	32/S1-T6	19	18	14	0
Nbs2-Rdg2a	41/S1-T7	23	1	17	0
	42/S1-T7	19	1	16	0
	46/S1-T7	16	0	9	0
	54/S1-T7	21	0	4	0
	56/S1-T7	17	1	5	0
	57/S1-T7	26	0	12	0
	60/S1-T7	20	2	16	0
	62/S1-T7	16	0	18	0
	64/S1-T7	17	0	7	0
	71/S1-T7	24	0	16	0
Thibaut (*Rdg2a*)		40[c]	38	6	0
NIL3876 (*Rdg2a*)		35	34	25	0
Mirco (*rdg2a*)		35	0	19	0
Golden Promise (*rdg2a*)		35	2	9	0
15/S1-T6 (empty vector)		36	1	15	0

[a]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.
[b]Number of transgenic T_1 plants without leaf stripe symptoms. Data were pooled from three independent experiments each comprising 5 or more plants per line.
[c]Total number of plants tested as controls.

Tab 3.1 Complementation test of leaf stripe susceptibility in the barley cv. Golden Promise (Bulgarelli *et al.*, 2010).

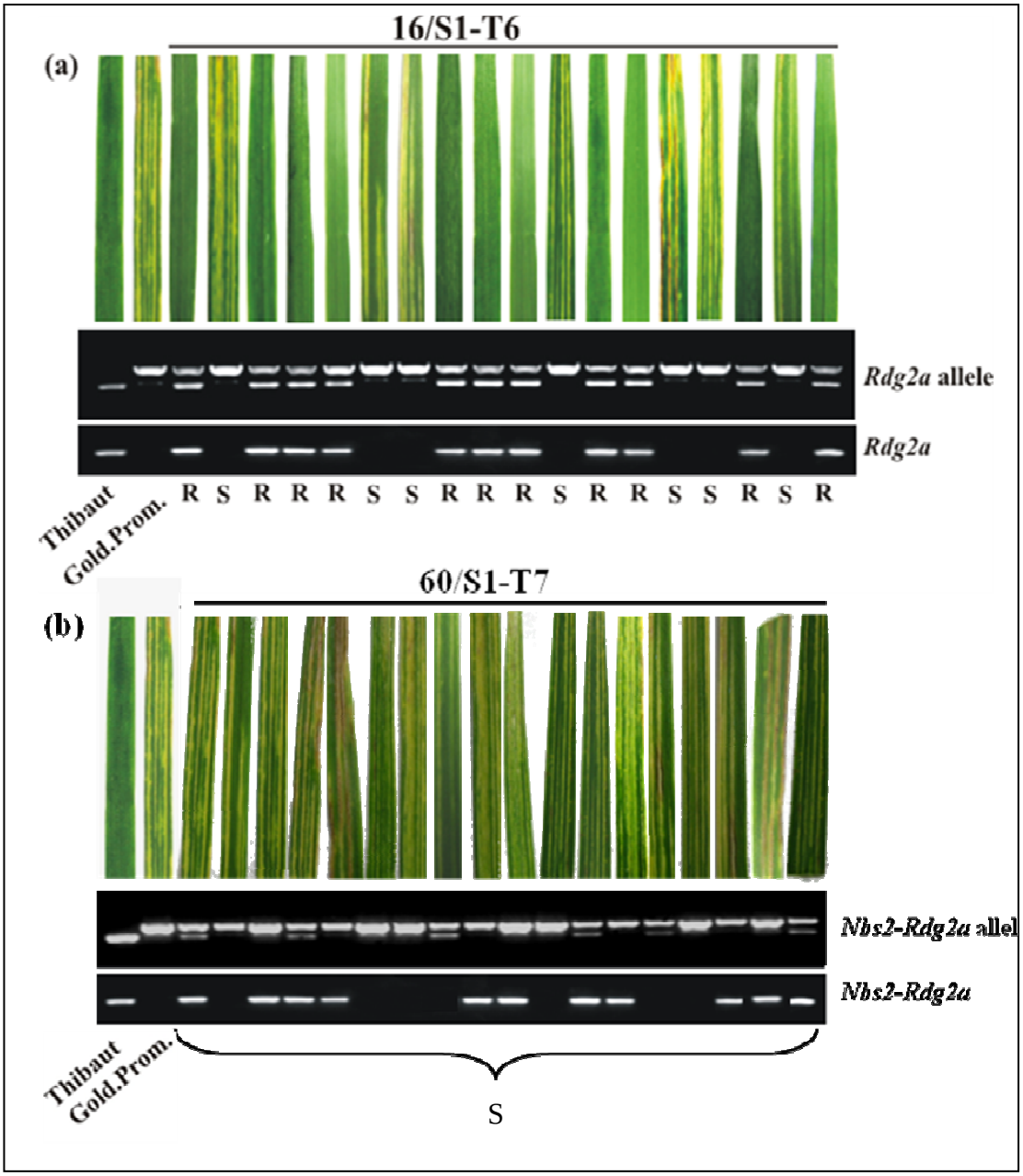


Fig. 3.6 (a) Segregating T_1 seeds of the transgenic line 16/S1-T6, carrying the *Nbs1-Rdg2a* transgene, were inoculated with *P. graminea* isolate *Dg2* and the plants were analyzed for resistant/susceptible phenotype and by an *Rdg2a* STS marker allele. The same lines were also analyzed for transgene expression using RT-PCRs. The resistant cv. *Thibaut* and the susceptible cv. *Golden Promise* are shown as controls (Bulgarelli *et al.*, 2010). **(b)** The same analysis conducted on T_1 seeds of the transgenic line 60/S1-T7 in which the *Nbs2-Rdg2a* gene was introducted.

Fig. 3.7 shows a Southern blot analysis carried out using genomic DNAs extracted from several *Dg2*-resistant S1-T6 lines, carrying the *Nbs1-Rdg2a* transgene, and hybridized with a probe specific for Thibaut *Nbs1-Rdg2a*. Thibaut and Golden Promise genomic DNAs were used as references. All the transgenic lines showed

the Golden Promise pattern with an addition of the Thibaut hybridizing fragment, thus confirming the presence of the transgene. In particular, line 8 showed more intense signals with respect to Thibaut and lines 16 and 32 had signals about three times stronger than Thibaut. Probably these lines have a higher copy number of the transgene with respect to Thibaut, but more accurate analyses are necessary to evaluate the exact copy-number.

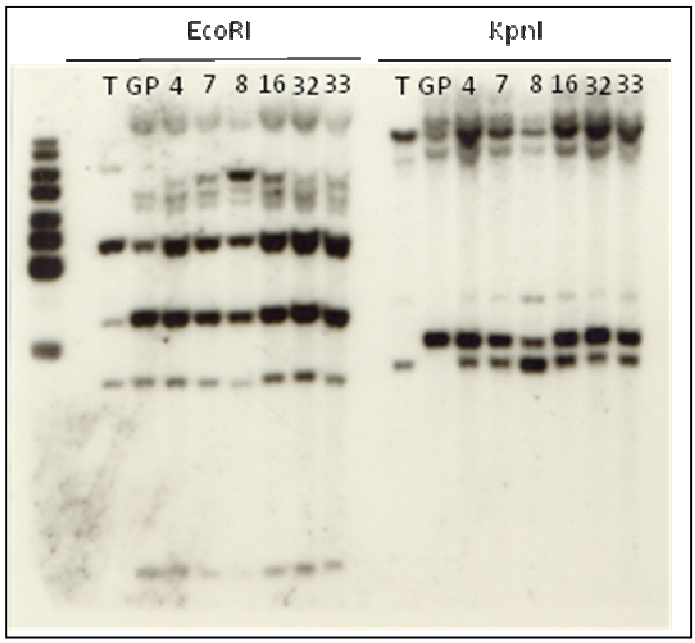


Fig. 3.7 Southern blot analyses of several resistant S1/T6 plants carrying the *Nbs1-Rdg2a* transgene. Genomic DNAs were digested by EcoRI and KpnI. The probe was specific for the gene and was obtained from Thibaut *Nbs1-Rdg2a* by PCR. The corrispective numbers of the lines are reported. Thibaut (T) and Golden Promise (GP) were used as reference controls.

The *Rdg2a* gene confers resistance by arresting fungal growth at the scutellar node and basal region of the provascular tissues of barley embryos (Haegi *et al.*, 2006). RT-PCR analyses using RNA extracted from the leaves of some resistant and susceptible 16/S1-T6 plants grown in the absence of *P. graminea* (control) and inoculated with isolate *Dg2* and *Dg5* were performed. Primer pairs were specific for fungal *Ubiquitin* and *GTPase activator* and Thibaut *Nbs1-Rdg2a*. Barley *Actin* was used as the reference gene. 16/S1-T6-*Rdg2a* plants infected with *Dg2* (16/S1-T6-P5-*Rdg2a-Dg2*) showed no leaf stripe symptoms and no fungal mycelium in the leaves, as demonstrated by the absence of transcripts for fungal *Ubiquitin* and *GTPase activator* genes. Typical leaf stripe symptoms and fungal transcripts were instead

present in leaves of compatible interactions (16/S1-T6-*rdg2a* plants infected with isolates *Dg2* and *Dg5* (16/S1-T6-P2-*rdg2a-Dg2* and 16/S1-T6-P3-*rdg2a-Dg5*) and 16/S1-T6-*Rdg2a* plants infected with *Dg5* (16/S1-T6-P6-*Rdg2a-Dg5*)) (Fig. 3.8).

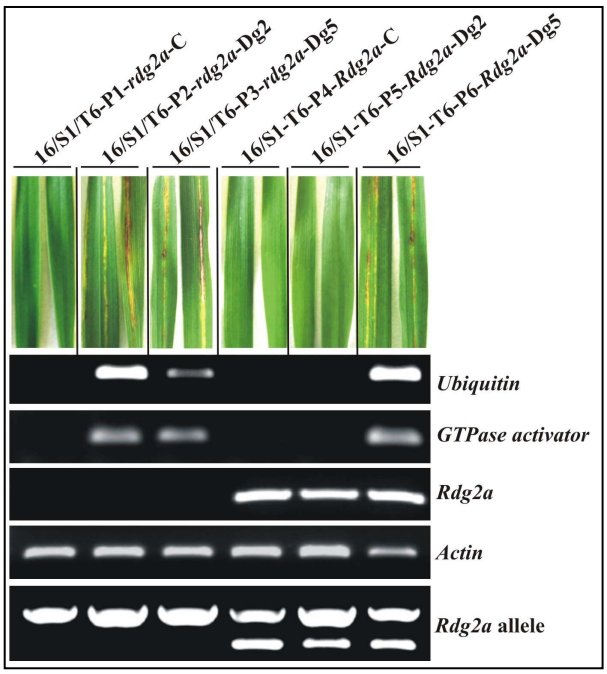


Fig. 3.8 RT-PCR analyses of the fungal *Ubiquitin* and *GTPase activator* genes and of the Thibaut *Rdg2a* gene in six different 16/S1-T6 plants (P1 to P6) segregating for the *Rg2a* transgene. Barley *Actin* gene was used as an internal control. The DNA extracted from the same plants was tested for the presence of the *Rdg2a* allele at an *Rdg2a* STS marker (Bulgarelli *et al.*, 2010).

These findings further confirm that *Nbs1-Rdg2a* represents the *Rdg2a* gene.

3.5 The RDG2A protein

The predicted RDG2A protein consists of 1,232 amino acids and has an estimated molecular weight of 139.73 KDa. It contains all the conserved NB domain motifs of the NB-LRR proteins defined by Meyers *et al.* (1999; 2003), including the P-loop, RNBS-A, GLPL, RNBS-D and MHD domains, the latter of which is duplicated (Fig. 3.9). A COILS analysis revealed the presence of a potential *C*oiled-*C*oil (CC) domain between amino acids 25 and 60, indicating that RDG2A belongs to the group of the CC-NB-LRR protein family (Meyers *et al.*, 1999). The LRR region contains 22 imperfect repeats with a few repeats showing good agreements with the consensus motif LxxLxLxx(C/N/T)/xxLxxLxxLP for cytoplasmic LRRs (Fig. 3.9) (Jones and Jones, 1997).

Fig. 3.9 The RDG2A protein domains. The predicted CC domain is underlined. Motifs conserved in the NB region of the NB-LRR proteins are in blue, and are (in order): P-loop, RNBS-A, Kinase 2, RNBS-C, GLPL, RNBS-D and MHD. Amino acids conforming to the cytoplasmic LRR consensus LxxLxLxx(C/N/T)/xxLxxLxxLP are in red. CT denotes the RDG2A C-terminal region (Bulgarelli *et al.*, 2010).

Fig. 3.10 illustrates a phylogenetic tree obtained using RDG2A and the most similar sequences present in the *N*ational *C*enter for *B*iotechnology *I*nformation (NCBI) database. Searching was conducted using BLASTp, the sequences were aligned by ClustalX and the alignment was visualized by GeneDoc. The tree was created using

the Phylip software with the Neighbor-joining algorithm and visualized by Treeview. RDG2A was most similar (47-52%) over its whole length to five rice resistance-like proteins (BAD08990, EEE69085, EEC83970, BAD0894, and BAF24312) 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 collinear with the barley *Rdg2a* interval (Bulgarelli *et al.*, 2004). Similarities with known barley resistance proteins (MLA1, MLA6 and MLA12 powdery mildew resistance proteins) are restricted to the conserved motifs of the NB domain (low level of identity, approximately 16%) (Fig. 3.10).

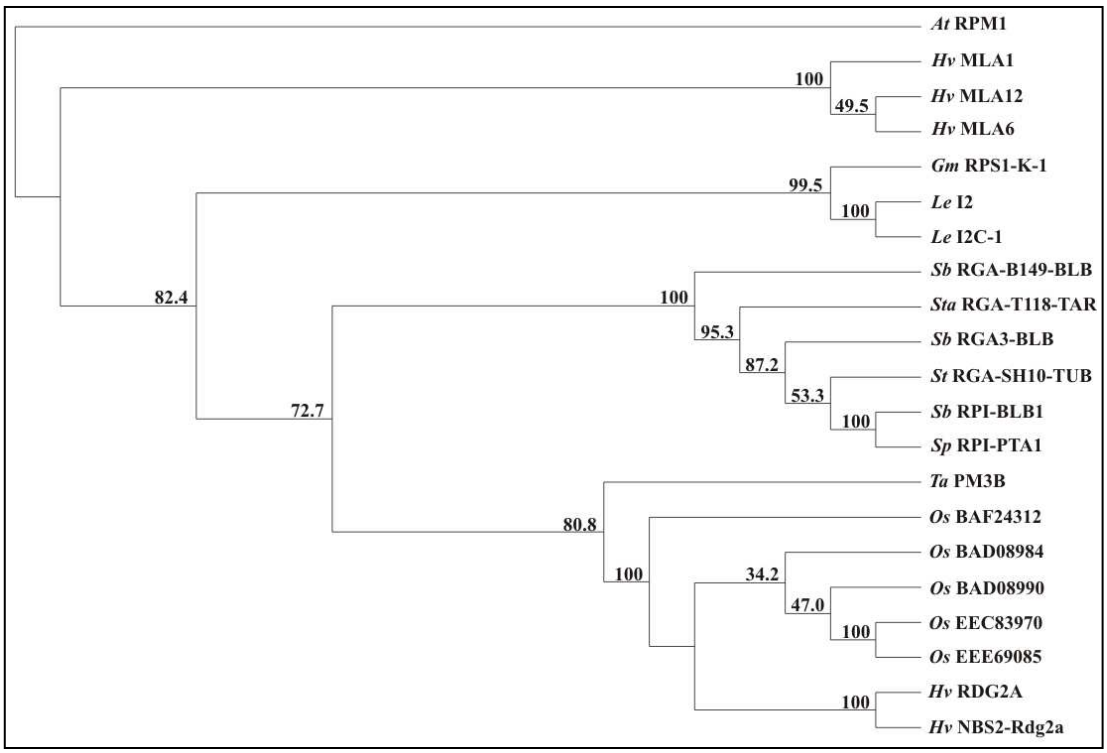


Fig. 3.10 Neighbor-joining phylogenetic tree including RDG2A, similar resistance proteins and resistance gene analog products. Numbers on branches indicate bootstrap percentages; while prefixes indicate species origin. The *A. thaliana* RPM1 protein (Q39214) was used as outgroup. Shown are the rice (*Oryza 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 the *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 (Bulgarelli *et al.*, 2010).

Comparison of the RDG2A (protein ID ADK47521) and NB2-RDG2A (protein ID ADK47522) sequences using ClustalW, showed that the two proteins are 73.5%

identical, and differences include a deletion of three consecutive LRRs in NB2-RDG2A (Fig. 3.11). Similarity is higher in the CC region than in the NB and LRR domains (92.6% versus 73-74%), and the proportion of non-conservative amino acids substitutions is lower in the NB domain (75/104=72%) than in the LRR domain (57/71=80%). Similarly, the ratio of non-synonymous (*Ka*) to synonymous (*Ks*) 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 β-strand/β-turn xxLxLxx motifs (solvent-exposed residues framed by aliphatic residues (Jones and Jones, 1997)) (boxed Fig. 3.11) than elsewhere (25/133=18.8% versus 32/373=8.5%).

These comparisons indicate that *Rdg2a* and its paralogues have been subjected to diversifying selection in the LRR-coding region, consisting with the fact that the LRR domain is an important determinant for resistance specificity (Bulgarelli *et al.*, 2010; DeYoung, 2006).

Fig. 3.11 Alignment of the deduced LRR domain sequences of RDG2A and NB2-RDG2A. Substitution differences are boxed; those in grey and green represent conservative and non-conservative substitutions (as defined by ClustalW), respectively. The regions of the LRRs that correspond to the β-strand/β-turn motif xxLxLxx are framed and the Leucine (or other aliphatic) residues that form the structural backbone of the LRR units in RDG2A are in red (Bulgarelli *et al.*, 2010).

Transiently expression in barley cv. Golden Promise leaf epidermal cells of RDG2A and NB2-RDG2A fused with the *Y*ellow *F*luorescent *P*rotein (YFP) at their N-terminal showed that the two proteins are localized in the nucleus and in the cytoplasmatic strands, even if they don't have any predicted transmembrane domain or any signal peptide sequence (Fig. 3.12) (Bulgarelli *et al.*, 2010).

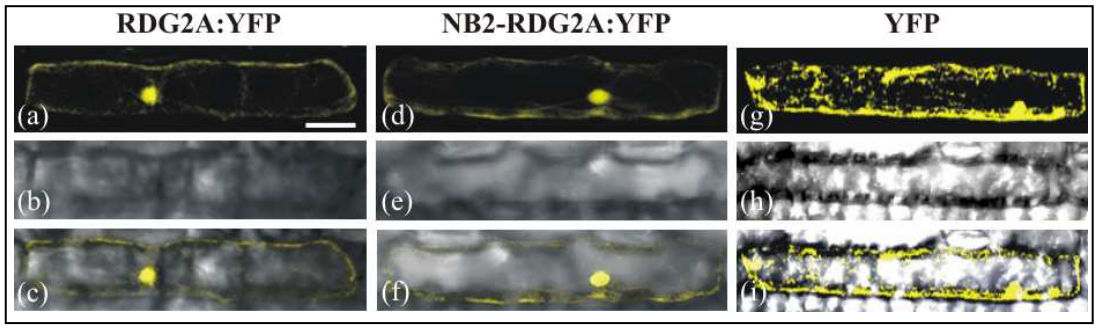


Fig. 3.12 Sub-cellular localization of the RDG2A and NB2-RDG2A proteins. Barley cv. Golden Promise epidermal cells were transiently transformed with constructs expressing RDG2A:YFP and NB2-RDG2A:YFP fusion proteins (**(a)** and **(d)** respectively), driven by the maize *Polyubiquitin* gene promoter. **(g)** control construct expressing YFP alone with the same promoter. 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 (Bulgarelli *et al.*, 2010).

3.6 *Rdg2a*-mediated resistance does not involved programmed cell death

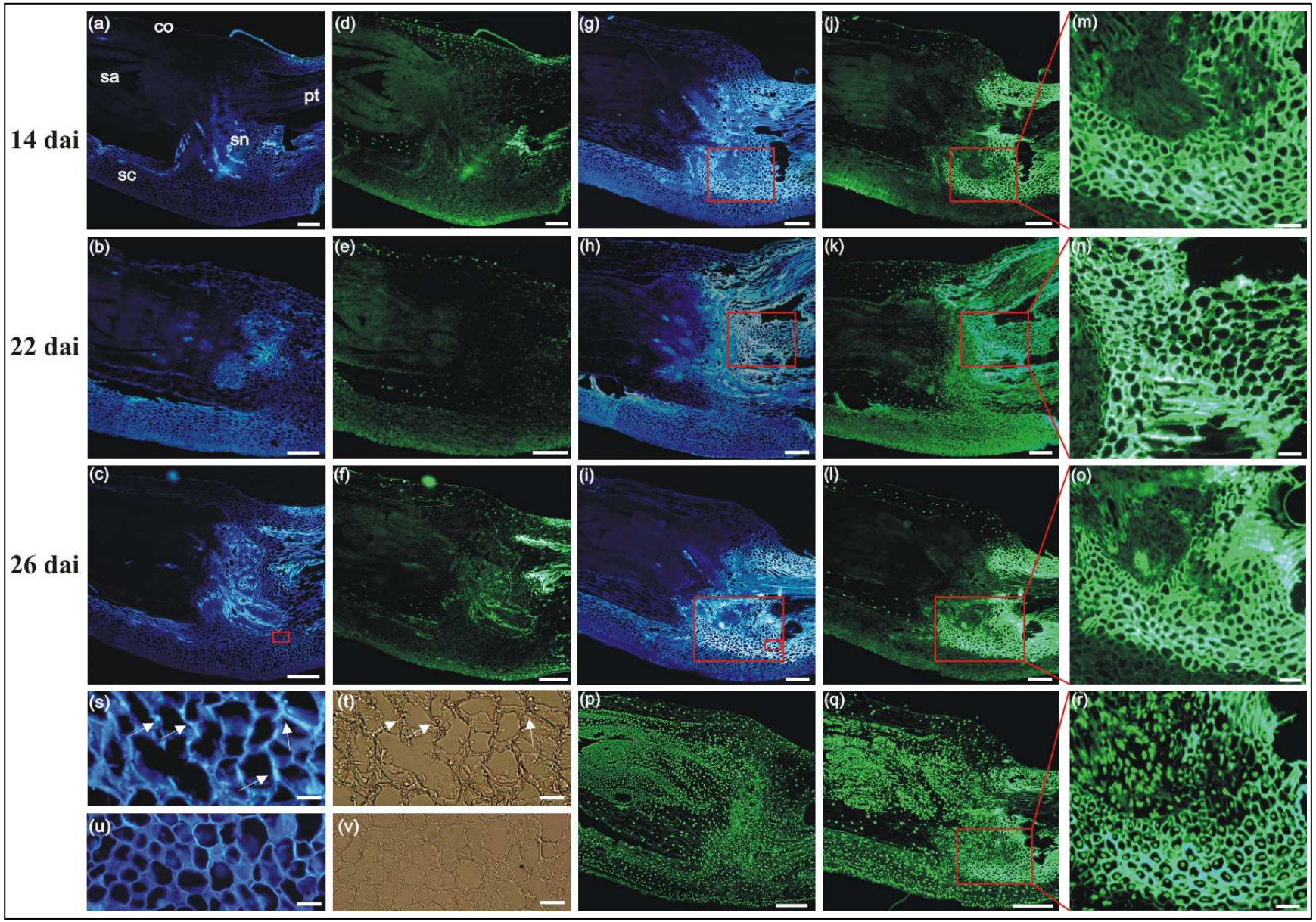
Redg2a-mediated resistance terminates fungal growth, in inoculated embryos, through the reinforcement of cell wall by the accumulation of phenolic compounds with the appearance of cell wall-associated host-cell autofluorescence at the junction of the scutellum and the scutellar node (Haegi *et al.*, 2008). Whole-cell autofluorescence is regarded as an indicator of *H*ypersensitive *R*esponse (HR) in race-specific resistance of barley leaf epidermal cells to powdery mildew (Huckelhoven *et al.*, 1999; Gorg *et al.*, 1993) but it was only occasionally (one or two cells per embryo section) observed in barley embryos expressing *Rdg2a* resistance. To analyse whether this resistance involves HR and so *P*rogrammed *C*ell *D*eath (PCD), we conducted a TUNEL (*T*erminal Deoxynuclotidyl *T*ransferase-mediated d*U*TP *N*ick and *L*abelling) test on serial sections of NIL3876-*Rdg2a* barley

embryos. This method enables the detection of free 3'-OH groups generated by DNA strand breaks occurring at the first stage of programmed cell death. Embryos inoculated with *P. graminea* isolate *Dg2* for 14, 22 and 26 days and control embryos, grown in the absence of the fungus at the same time-points, were treated with TUNEL reaction and examined under UV light. Autofluorescence was also investigated using a different epifluorescence filter. To verify the presence of the fungus in inoculated embryos, sections were incubated in Calcofluor staining.

In non-inoculated embryos, no autofluorescence was observed (Fig. 3.13(a) to (c)), while inoculated embryos showed autofluorescence at the scutellar node and provascular tissues (Fig. 3.13(g) to (i)).

Clalcofluor staining and bright field observations revealed the presence of fungal mycelium in the autofluorescent regions (Fig. 3.13(s) and (t), respectively), indicating that autofluorescence is a genuine defence-associated marker.

The TUNEL analysis revealed some nuclear DNA fragmentation (bright green fluorescent nuclei) in the coleoptiles and in a few cells at the scutellar node of both control (Fig. 3.13(d) to (f)) and inoculated (Fig. 3.13(j) to (l) and (m) to (o)) embryos. There was no difference in TUNEL signals at the junction of the scutellum and scutellar node between inoculated and control embryos and the presence of positive nuclei was only occasionally (one/two nuclei over 500 cells) (Fig. 3.13(d) to (f), (j) to (l) and (m) to (o)). Following the treatment of control and inoculated embryos with DNaseI (positive control), all nuclei in all embryo tissues showed positive signals both in the presence and absence of the fungus (Fig. 3.13(p) to (r)). As expected, no signals were observed in sections in which deoxynucleotidyltransferase enzyme was omitted (negative control; data not shown). This observations suggested that the analysis worked effectively.


14 dai
22 dai
26 dai
(a)
co
sa
pt
sn
sc
(b)
(c)
(d)
(e)
(f)
(g)
(h)
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(u)
(v)

Fig. 3.13 Histological analyses of NIL3876-*Rdg2a* barley embryos. **(a) to (c)** Autofluorescence of sections of control embryos grown for 14 (a), 22 (b) and 26 (c) days in the absence of *P. graminea*. **(d) to (f)** TUNEL analysis of sections in (a) to (c). **(g) to (i)** Autofluorescence of sections of embryos inoculated with leaf stripe isolate *Dg2* for 14 (g), 22 (h) and 26 (i) days. **(j) to (l)** TUNEL test of sections in (g) to (i). **(m) to (o)** Magnified views of the red boxes in (j) to (l) and (g) to (i). **(s) and (t)** Magnified views of the small red box in (i) stained with calcofluor observed under UV light (s) or under bright field (t); arrows indicate the intercellularly growing *P. graminea* mycelium. **(u) and (v)** Magnified views of the small red box in (c) stained with calcofluor observerved under UV light (u) or under bright field (v). **(p) and (q)** Respectively, sections of control and inoculated embryos at 26 dai, treated with DNaseI and subjected to TUNEL analysis. **(r)** Magnified view of the red box in (q). Scale bars represent 200 µm (a) to (l), 50 µm (m) to (o) and 25 µm (s) to (t). co=coleoptiles, pt=provascular tissue, sa=shoot apex, sn=scutellar node (Bulgarelli *et al.*, 2010).

Staining with 4'6-diamino-2-phenylindole dihydrochloride (DAPI), indicated the presence of undamaged nuclei in all the sections and in particular in autofluorescent regions (Fig. 3.14), confirming that the absence of TUNEL signal was not due to the absence of the nucleus in cells.

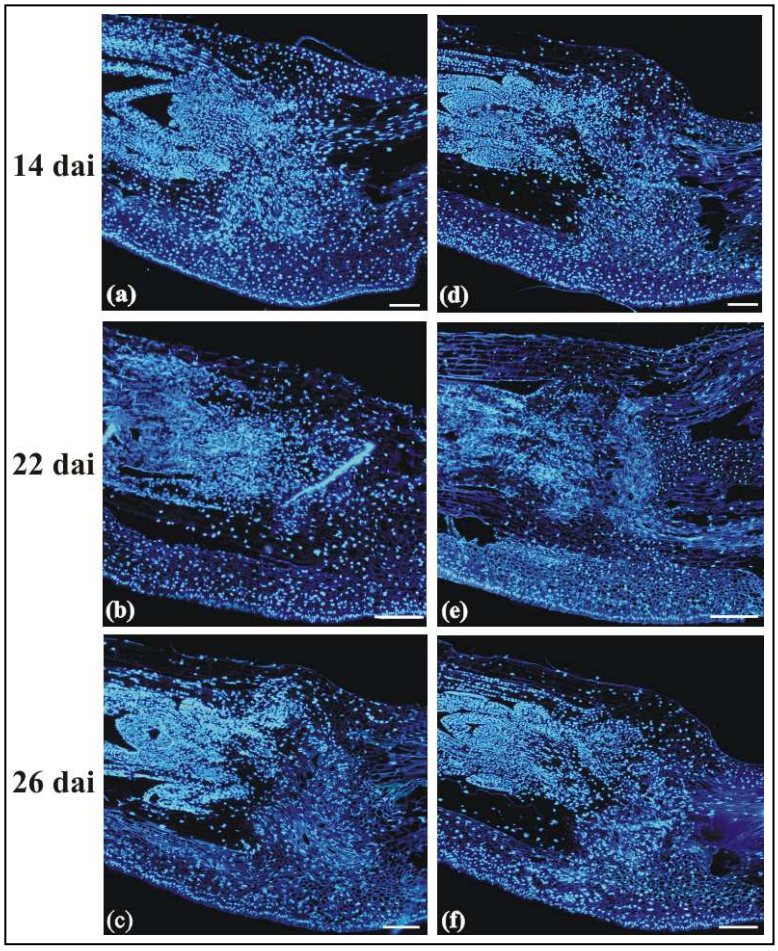


Fig. 3.14 DAPI staining of embryo sections analyzed for autofluorescence and by TUNEL test in Fig. 3.13. **(a) to (f)** correspond to section in Fig. 3.13(a) and (d), (b) and (e), (c) and (f), (g) and (j), (h) and (k), (i) and (l), respectively (Bulgarelli *et al.*, 2010).

The observation that there were no significant differences in TUNEL signals between inoculated and control embryos demonstates that the *Rdg2a*-mediated resistance does not involve PCD.

3.7 Analysis of the *rdg2a* locus in barley cultivar Morex

Steuernagel *et al.* (2009) conducted a *de novo* 454 sequencing of 91 barcoded, pooled BACs from barley cv. Morex and assembled a 26,223 bp-long contig belonging to the HVVMRXALLhA425O23_c2 BAC (mwg7_ HVVMRXALLhA425O23_c2 contig; supplementary materials). Fig. 3.15(a) shows the comparison between this contig and the Thibaut 72,645 bp-long contig (accession number HM124452), performed using the BLAST (blast2seq) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In the two genotypes several regions showed a high level of sequence similarity but also rearrangements consisting in deletions at the level of Morex sequence (Fig. 3.15(a)).

The correct assembly of the mwg7_HVVMRXALLhA425O23_c2 contig was verified using a PCR-based molecular markers analysis with different primers that annealed to sequences within the deleted regions or flanking them (Fig. 3.15(a)) (primer combinations and expected amplicon dimensions are listed in Tab. 2.7, Par. 2.7.1, Materials and methods) and comparing the results obtained from Thibaut genomic DNA with the a Morex BAC (146G20). On the basis of PCR analyses, the Morex BAC 146G20 was demonstrated to overlap within the mwg7_HVVMRAXALLhA425o23_c2 BAC sequence (data not shown). All the primer combinations gave the expected results in terms of size and sequence of the amplicons (Fig. 3.15(b)), thus confirming that the assembling of the Morex contig was correct. This analysis was also carried out on Morex genomic DNA and provided the same results as those obtained for the BAC 146G20 (data not shown).

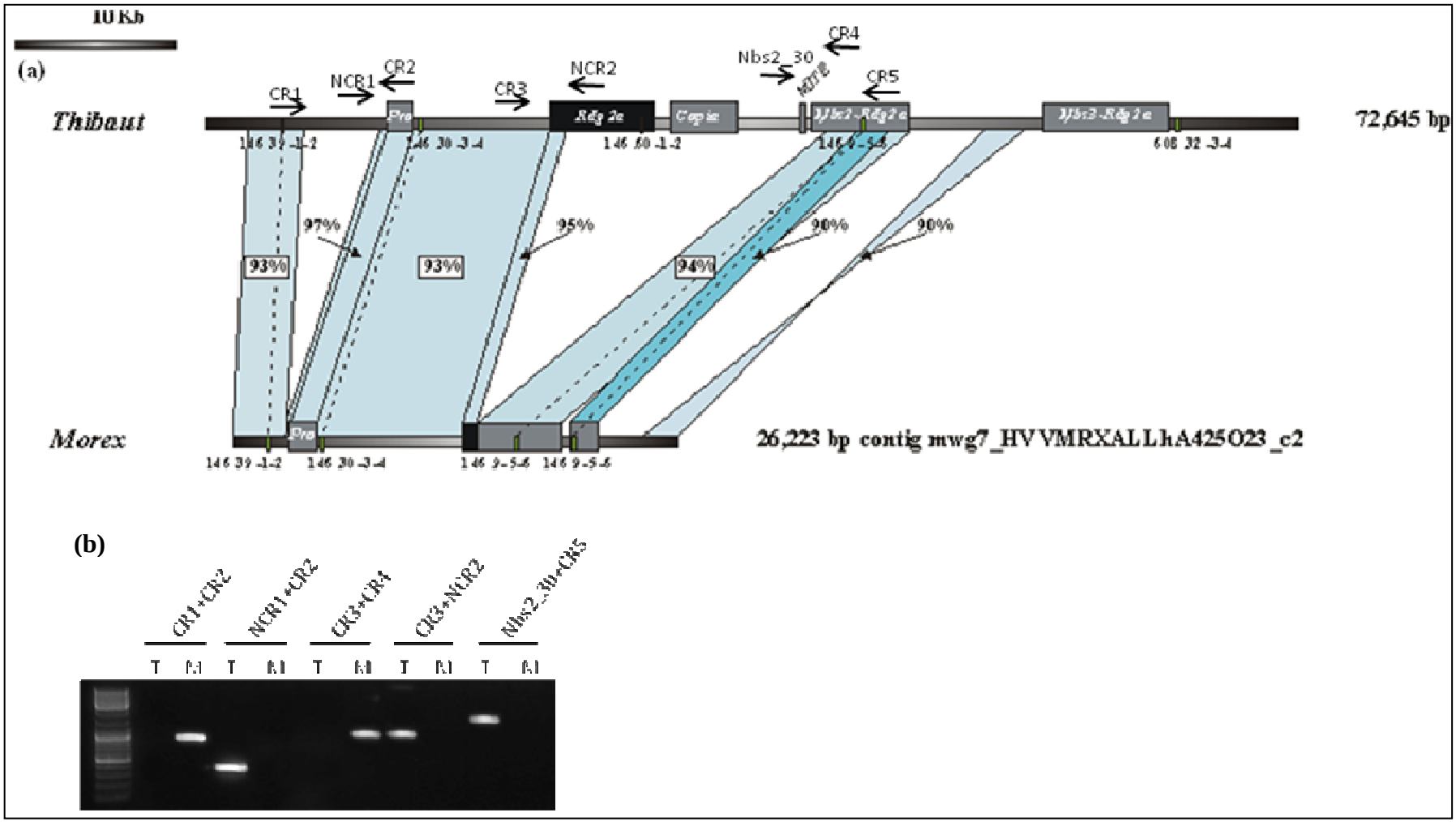
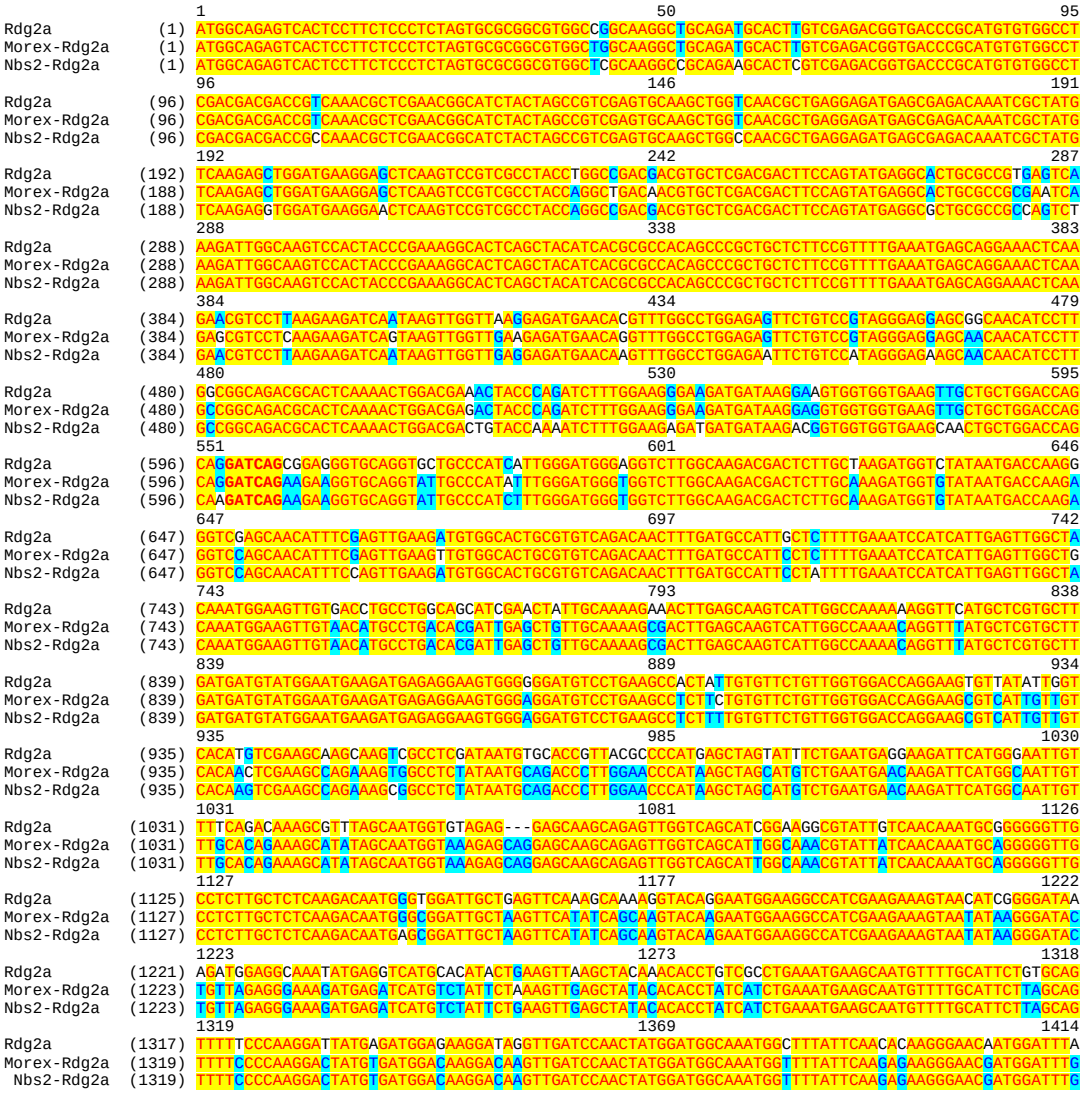
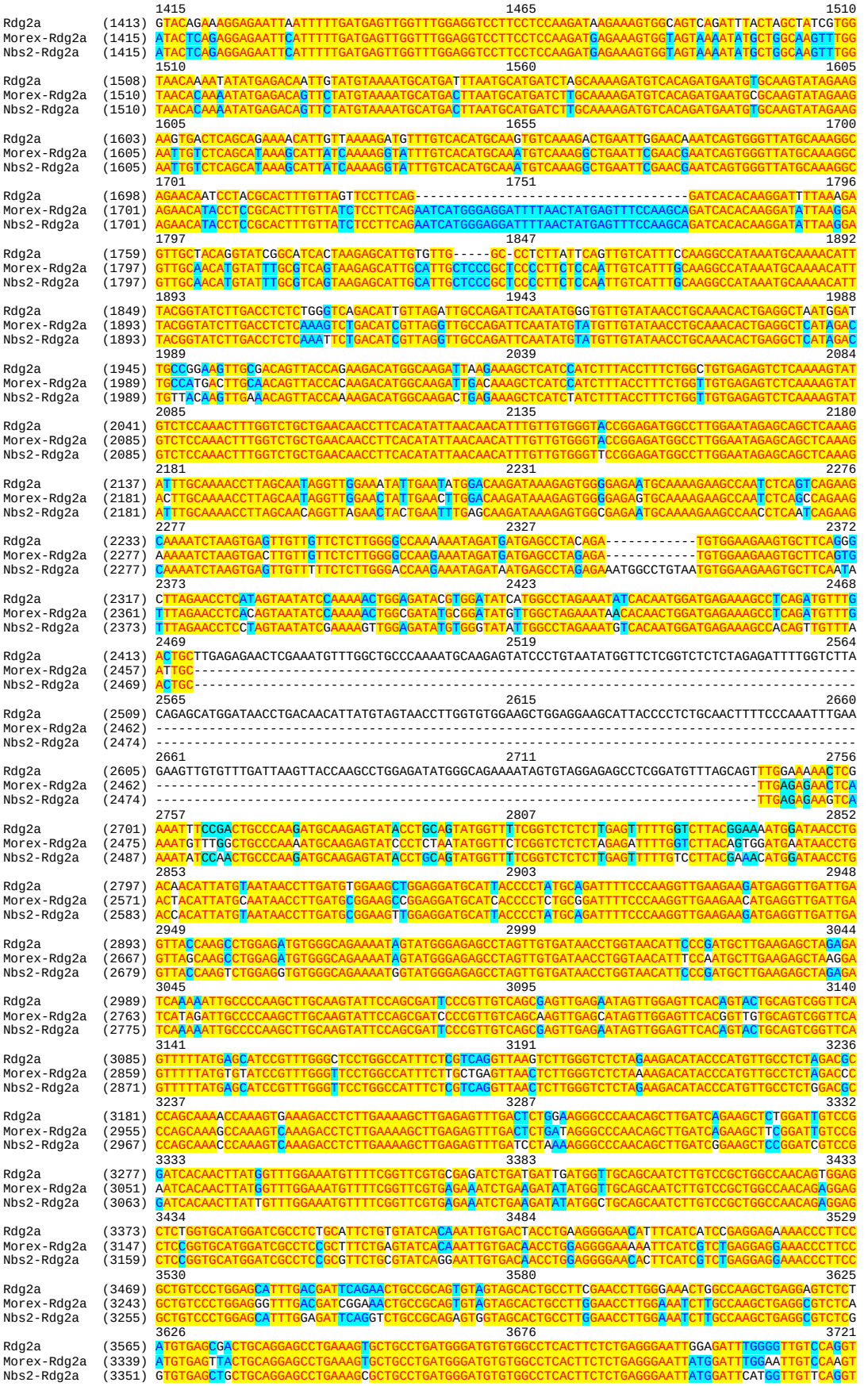


Fig. 3.15 (a) Comparison between the Morex 26,223 bp contig (mwg7_HVVMRXALLhA425O23_c2) and the Thibaut 72,645 bp contig. All genes are indicated and the percentages of identity among Morex and Thibaut synthenic regions are reported. Arrows represent the positions of the primers used for the PCR-based molecular markers analyses. **(b)** PCR-base molecular markers analysis carried out on Thibaut genomic DNA (T) and Morex BAC 146G20 (M). Primers combinations utilized are reported above the gel.

By considering the highest level of similarity and informative polymorphisms highlighted from multiple alignement of the coding sequences (Fig. 3.16), Morex sequence for the *Rdg2a* allele is apparently derived from a *Rdg2a*-homolog sequence, for the putative regulatory region and the first 555 bp of the coding sequence (a region that encompass the CC domain and 108 bp encoding for the NB domain); while from the base 562 until the end of the transcribed region (including the rest of the NB domain and the LRR domain encoding sequences) the derivation is from a *Nbs2-Rdg2a*-homolog sequence (Fig. 3.17). In *Nbs2-Rdg2a*, a deletion of 201 bp with respect to *Rdg2a* is observed from position 2,397 bp to position 2,598 bp of *Rdg2a*. This resulted in the lacking of three LRR units at the level of the encoded protein. Such deletion is also present in Morex *Rdg2a* allele.





```
                    3722                                                3772                                                3817
Rdg2a         (3661) ATGGAGGAATTCCCGCATGGTCTCCTGGAGCGGTTGCCAGCCCTCGAATACTGTAGCATCCATCTCTGCCCGGAGTTGCAAAGACGATGCAG
Morex-Rdg2a   (3435) ATGAAGAAATTCCCGCATGGTCTCCTAGAGCGGTTGCCAGCTCTCGAACACTTGAGCATACATGACTGCCCGGAGTTGGGAACACGATGCAG
Nbs2-Rdg2a    (3447) ATGGAGGAATTCCCGCATGGTCTCCTGGAGCGGTTGCCAGCCTCGAATCCTTTAGCATACGTGGCTGCCCGGAGTTGGGAAGACGATGCGG
                    3818                                                3868                                                3897
Rdg2a         (3757) AGAAGGTGGGGAGTACTTCCACTTGCTCTCCTCTGTTCCACGTAAATACTTTGAGAGAATAGGCATCCCAAAGTGA
Morex-Rdg2a   (3531) AGAAGGTGGGGAGTATTTCCACTTGCTCTCCTCTGTCCCACGTAAAGACATTTCGCGATGA---------------
Nbs2-Rdg2a    (3543) AGAAGGTGGGGAGTATTTCCACTTGCTCTCCTCTGTCCCACGTAAAGACTGTTAG--------------------
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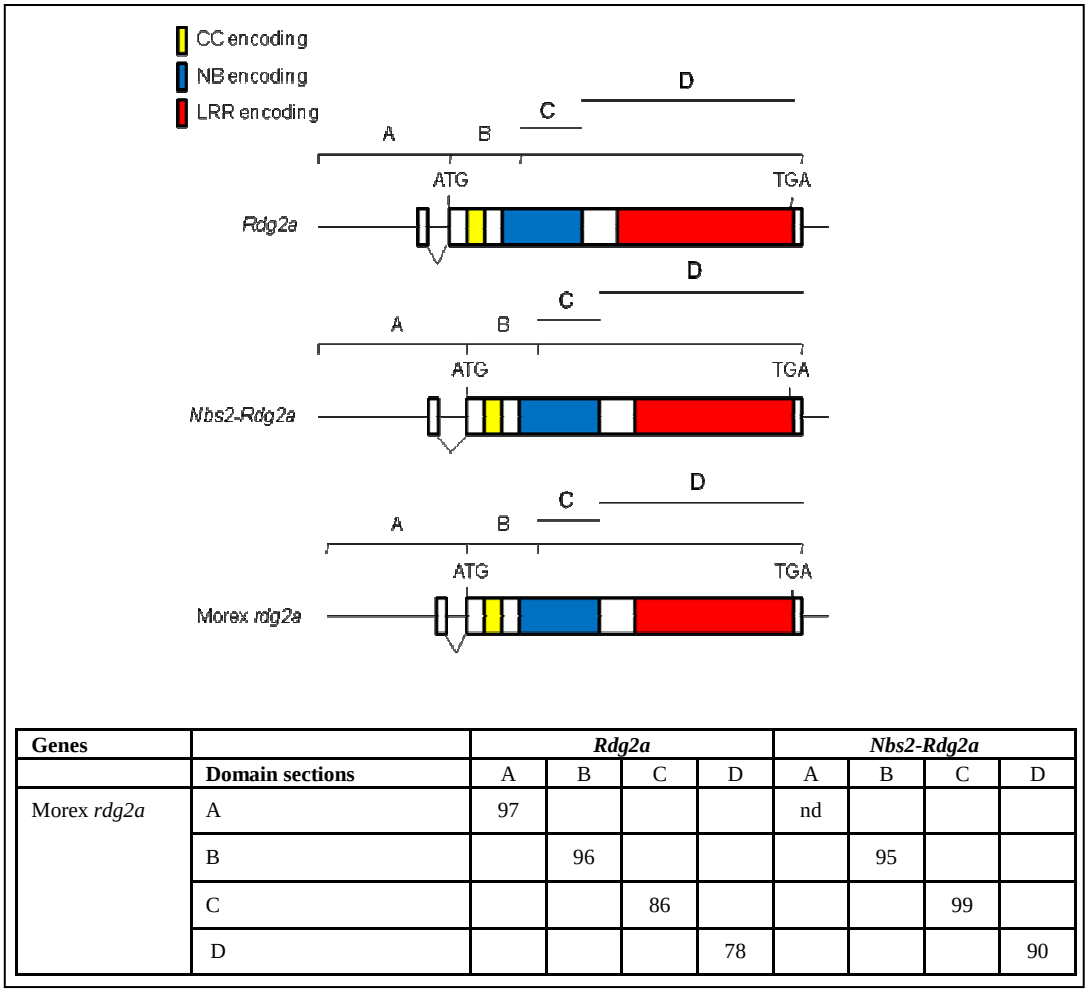
Fig. 3.16 Alignment between the three genes: Thibaut *Rdg2a*, Thibaut *Nbs2-Rdg2a* and Morex *rdg2a*. Regions conserved in all genes are highlighted in yellow; regions conserved in only two genes are highlighted in light blue.



Genes		*Rdg2a*				*Nbs2-Rdg2a*			
	Domain sections	A	B	C	D	A	B	C	D
Morex *rdg2a*	A	97				nd			
	B		96				95		
	C			86				99	
	D				78				90

Fig. 3.17 DNA sequence similarities between Morex *rdg2a* and Thibaut *Rdg2a* and *Nbs2-Rdg2a*. Diagrams above define the domains compared. Percentages of identity are reported. nd=not determined.

These results support the possibility that Morex *Rdg2a* allele is derived from unequal crossing-over between ancestral *Rdg2a* and *Nbs2-Rdg2a* members of the gene family. This crossover led to the elimination of part of the *Rdg2a*, the Copia-like retrotransposone and a portion of the 5'end of *Nbs2-Rdg2a*, the reduction of the members of the gene family and and the generation of to the Morex allele, whose

coding sequence is derived only partially from *Rdg2a*, while for the remaining coding sequence is originated from *Nbs2-Rdg2a*-like sequence.

The third member of the gene family (as identified in the Thibaut genomic sequence), namely as *Nbs3-Rdg2a*, did not match within the available Morex contig with a significant homology; but because only about 26 Kb of the Morex contig were available in the BAC contig mwg7_HVVMRAXALLhA425o23_c2, it was not possible to exclude the presence of this gene in Morex genomic background.

PCRs using primer combinations specific for *Nbs3-Rdg2a* were therefore carried out on Morex BAC 146G20 and an amplicon of about 3 Kbp was obtained using a forward primer designed at the end of the mwg7_HVVMRAXALLhA425o23_c2 BAC contig and a reverse primer designed at the 5'end of *Nbs3-Rdg2a*. The comparison of the amplicon sequence with Thibaut *Nbs3-Rdg2a* showed 90% of sequence identity in the overlapping regions, demonstrating the presence of an *Nbs3-Rdg2a* allele in the cv. Morex. The complete sequencing of this member of the gene family in Morex is still in progress.

To analyze the expression of Morex *rdg2a*, a two-step reversetranscription PCR was performed, using gene-specific primer, on DNaseI treated RNA extracted from Morex control embryos and from *P. graminea* isolate *Dg2* inoculated embryos at 7, 14 and 22 dai. RNA was also axtracted from leaves of 14 days old Morex seedlings infected with the fungus and from control leaves. Barley *Actin* gene was used as the reference gene. After amplification the identity of the amplicons was checked by sequencing. The gene was found to be transcribed in both embryos tissues and leaves (Fig. 3.18(a)).

To complete the expression analysis, a quantitative RT-PCR was performed using RNAs obtained from Morex embryos as above. The experiment was carried out with three technical replicates for each RNA sample. As described in Par. 3.3, results were expressed as relative transcription of each gene, normalized to the expression of barley *Actin*, compared to the expression of the same gene in unchallenged conditions. Standard deviation was considered to define if the differences of expression among the different conditions were statistically significant. Following leaf stripe inoculation, in embryo tissues, the expression of Morex-*rdg2a* did not change until 22 dai; after this time-point the transcription rate in the inoculated

samples increased approximatively 1.8 times with respect to control samples (Fig. 3.18(b)). In leaves, the Morex-*rdg2a* mRNAs accumulation was not pathogen-responsive (data not shown).

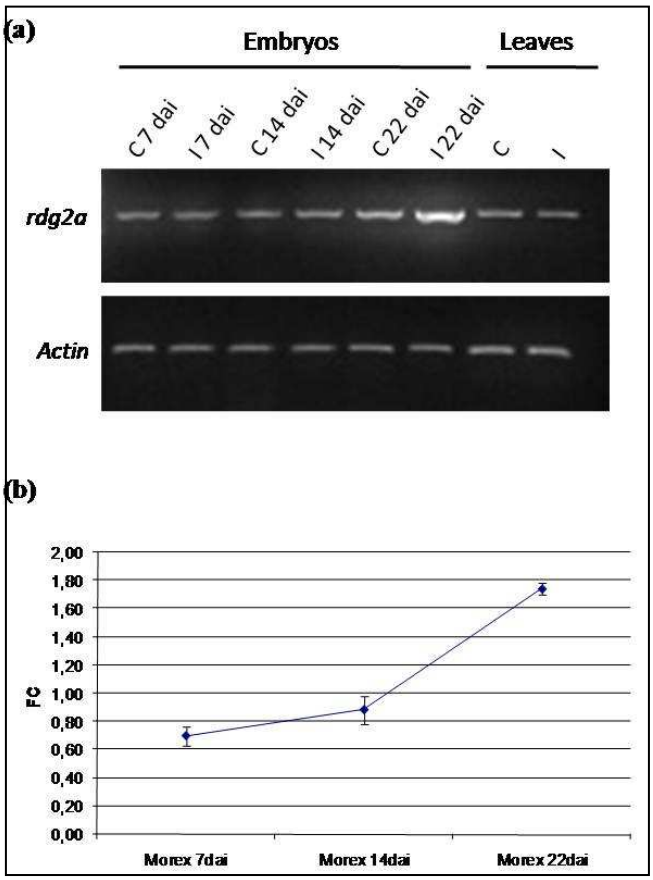


Fig. 3.19 (a) RT-PCR analysis of Morex-*rdg2a* under control and *P. graminea* isolate *Dg2* inoculation at three time-points (7, 14 and 22 dai) in embryo and in leaf tissues. Barley *Actin* gene was used as an internal control. **(b)** Quantitative RT-PCR at 7, 14 and 22 dai for Morex-*rdg2a*. Values are expressed as *F*old *C*hanges (FC) of transcript levels in the inoculated samples with respect to the transcript levels in uninoculated barley embryos. Error bars represent *S*tandard *D*eviation (SD) across all RT-PCR replicates (three for each sample).

Preliminary tests to determine whether Morex-*rdg2a* confers resistance to any *Pyrenophora graminea* isolates were carried out by infecting cv. Morex with seven different pathogen isolates (*Dg1*, *Dg2*, *Dg4*, *Dg10*, *Dg12*, *Dg19* and *Dg29*). Morex resulted to be fully resistant (0% of infected plants) to *Dg19* and *Dg29*. Nevertheless, it is not possible to ascribe a certain role to Morex-*rdg2a* in resistance to these isolates without performing complementation analyses transforming susceptible plants with this gene.

3.8 Haplotype analysis of the *Rdg2a* locus in different barley varieties

Different barley cultivars (Rika, Bulbul, Triumph, Imber, Optic, Ansis, Gitane, Bonus, Ketos, Grete, Franka, Marado and Federal) were tested for their resistance to *P. graminea* isolates *Dg2* or *Dg5*. Morex, Mirco, Golden Promise and Thibaut were also tested as controls (Tab. 3.2).

	Isolates	
	Dg2	***Dg5***
Cultivars	**% infected plants**	**% infected plants**
Morex	100	100
Mirco	100	100
Golden Promise	73	100
Thibaut	0	100
Ketos	100	100
Gitane	56	87
Triumph	0	92
Rika	28	0
Franka	62,5	100
Grete	100	100
Federal	39	8
Imber	0	0
Ansis	23	21
Bul Bul	25	0
Bonus	73	47
Optic	17	86
Marado	95	58
Jaidor	100	50

Tab 3.2 Infection tests conducted on different barley cultivars with *P. graminea* isolates *Dg2* and *Dg5*.

To analyze the frequency of Thibaut or Morex haplotypes for the *Rdg2a* locus in different susceptible and resistant barley genetic backgrounds, the above mentioned and other cultivars, whose restance to the two isolates was known (Rebelle, Onice, Proctor, Alf, Diadem, Haruna Nijo, Galleon, Jaidor, Nudinka, Passport and Acuario), were subjected to a PCR-based molecular markers screening using the same primer combinations as those described in Fig. 3.15(a) (Par. 3.7). Tab. 3.3

summarizes the *P. graminea* response for all the barley varieties considered (Arru *et al.*, 2003; Paragraph 2.2, Materials and methods).

	Resistance or susceptibility	
Cultivars	***Dg2***	***Dg5***
Morex	S	S
Mirco	S	S
Golden Promise	S	S
Thibaut	R	S
Ketos	S	S
Gitane	partially S	S
Triumph	R	S
Rika	partially R	R
Franka	partially S	S
Grete	S	S
Federal	partially R	R
Imber	R	R
Ansis	R	R
Bul Bul	R	R
Bonus	partially S	R
Optic	R	S
Rebelle	R	R
Onice	R	partially R
Proctor	partially R	R
Alf	R	partially R
Diadem	R	nd
Haruna Nijo	R	S
Galleon	R	S
Nudinka	S	S
Passport	R	nd
Jaidor	S	partially S
Acuario	R	partially S
Marado	S	partially S

Tab 3.3 Summary of the responses to *P. graminea* isolates *Dg2* and *Dg5* of the barley cultivars analyzed for the haplotype analysis of *Rdg2a* locus. nd=not determined.

Data are shown in Tab. 3.4.

Cultivars	Haplotype	Primer combinations				
		CR1+ CR2	NCR1+ CR2	CR3+ CR4	CR3+ NCR2	Nbs2_30+ CR5
Thibaut		/	471bp	/	1183bp	1927bp
Morex		1064bp	/	1179bp	/	/
Rika	1	T	200bp	T	T	M
Bulbul	1	T	200bp	T	T	M
Ansis	1	T	200bp	T	T	M
Gitane	1	T	200bp	T	T	M
Bonus	1	T	200bp	T	T	M
Ketos	1	T	200bp	T	T	M
Grete	1	T	200bp	T	T	M
Franka	1	T	200bp	T	T	M
Marado	1	T	200bp	T	T	M
Federal	1	T	200bp	T	T	M
Jaidor	1	T	200bp	T	T	M
Alf	1	T	200bp	T	T	M
Diadem	1	T	200bp	T	T	M
Mirco	1	T	200bp	T	T	M
Nudinka	1	T	200bp	T	T	M
Proctor	1	T	200bp	T	T	M
Golden Promise	1	T	200bp	T	T	M
Passport	1	T	200bp	T	T	M
Triumph	1	T	200bp	T	T	M
Optic	2	T	T	T	900bp	T
Acuario	2	T	T	T	900bp	T
Galleon	2	T	T	T	900bp	T
Rebelle	2	T	T	T	900bp	T
Haruna Nijo	2	T	T	T	900bp	T
Onice	3	T	M	T	900bp	M
Imber	4	M	M	M	M	M

Tab 3.4 Haplotype analysis for the *Rdg2a* locus in different barley cultivars using polymorphic PCR-based molecular markers. Sizes of the amplicons are reported; /=no amplification; T=Thibaut haplotype; M=Morex haplotype; green=varieties resistant to both *Dg2* and *Dg5* isolates; red=varieties susceptible to both the isolates; light blue=varieties resistant to *Dg2* but susceptible to *Dg5*; yellow=varieties susceptible to *Dg2* but resistant to *Dg5*; blu=variety resistant to *Dg2*, while response to *Dg5* is not known.

In the regions amplified by CR1+CR2 and CR3+CR4 primer combinations, Thibaut haplotype is conserved in almost all the varieties, regardless of their phenotype for reaction to leaf stripe. For the NCR1+CR2 genetic interval, most cultivars showed a shorter amplicon with respect to Thibaut and in only two cultivars (Imber and Onice) Morex amplicon was present. Also CR3 and NCR2 primers generated, in some genotypes, a shorter amplicon than Thibaut and, for this marker, only Triumph conserved Morex haplotype. The last primer combination (Nbs2_30+CR5) provided Morex haplotype for almost all the cultivars (except for Optic, Acuario, Galleon, Rebelle and Haruna Nijo), regardless of their resistant or susceptible behaviour to

isolates *Dg2* or *Dg5*, thus confirming that probably this gene is not involved in the resistant response to *P. graminea* isolate *Dg2*.

Considering the test performed at all the five loci, it was possible to identify four different haplotypes. Interentingly, four genotypes resistant to isolate *Dg2* but susceptible to isolate *Dg5* (Optic, Galleon, Haruna Nijo and Acuario) showed a haplotype highly similar to Thibaut haplotype with the exclusion of a slightly shorter amplification yielded by the primer combination CR3+NCR2. Also Rebelle genotype demonstrated to belong to this haplotype group. The *Rdg2a* alleles present in Rebelle, Galleon and Haruna Nijo were actually chosen for the re-sequencing (Supplementary materials). PCRs were performed on genomic DNAs of these varieties using *Rdg2a* specific primers that amplified overlapping fragments of the gene and designed on Thibaut sequence. Amplicons were directely sequenced using the same primers as those used for the amplification and with additional primers for sequence walking. The *Rdg2a* alleles derived from the three genotypes showed a 99% of nucleotide identity compared to the Thibaut *Rdg2a* in multiple alignments. Rebelle and Thibaut *Rdg2a* coding sequences are 100% identical. Haruna Nijo allele showed nucleotide changes (from AGGA to GGAC) at the position +402 from the start codon. Galleon *Rdg2a* revealed to contain two SNPs: G to A at +1915 and +1931 from the ATG and a sequence change (from ATGGT to TTAGG) at position +3175. These nucleotide variations resulted in differences at the protein level. Galleon RDG2A shows an Asparagine, instead of an Aspartic acid, and a Lysine, instead of an Arginine, at positions 639 and 644, respectively, within the fourth LRR unit. Moreover, in the fourtheen LRR the Tyrosine-1058 and the Cysteine-1059 have changed in Methionine and Valine. Despite these changes are in the LRR units, they do not belong to the region β-strand/β-turn. In Haruna Nijo RDG2A the Lysine-134 and the Glutamic-135 acid have changed in two Arginines. These amino acids are located between the CC and the NB domains at about 50 residues before the beginning of the NB domain. Since the conserved CC and NB domains and the variable LRR domain are those mainly implicated in the protein function, also considering that all the changes involve hydrophilic amino acids, it is possible that the observed changes does not affect the RDG2A activity.

RT-PCR, conducted on cDNA synthesized using DNaseI treated RNA extracted from the leaves of these three barley varieties, showed that the *Rdg2a* alleles are transcribed in all of them (Fig. 3.20).

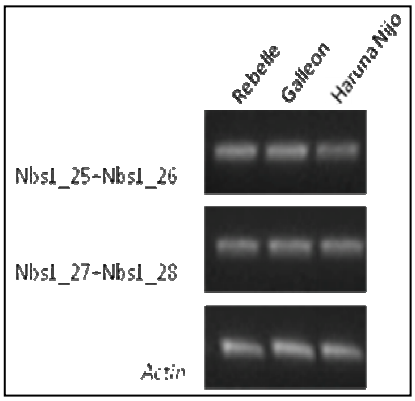


Fig. 3.20 RT-PCR analysis of *Rdg2a* in the three cvs. Rebelle, Galleon and Haruna Nijo resistant to isolate *Dg2*. The same primer combinations utilized for the RT-PCR in NIL3876 (Par. 3.3) were used. Barley *Actin* gene was the internal control.

4. Discussion

4.1 Identification of the *Rdg2a* resistance gene and evolution at the *Rdg2a* locus

4.1.1 The *Rdg2a* function is encoded by *Nbs1-Rdg2a*

As described in Introduction (Par. 1.3.1), the *Rdg2a* locus in the resistant cv. Thibaut was cloned and its sequencing led to the identification of three genes coding for CC-NB-LRR proteins (Accession number HM124452). These genes were called as *Nbs1-Rdg2a*, *Nbs2-Rdg2a* and *Nbs3-Rdg2a* (Bulgarelli *et al.*, 2010).

Based on these findings, the main question to be addressed was: "Which is the *Rdg2a* gene?".

To provide an answer, a comparison between the structures of the three candidates was first performed. *Nbs1-Rdg2a* and *Nbs2-Rdg2a* showed similar features: both were predicted as intronless genes and had coding sequences ranging from 3,477 bp (*Nbs2-Rdg2a*) to 3,699 bp (*Nbs1-Rdg2a*) that shared 82.1% of nucleotide identity (Bulgarelli *et al.*, 2010). *Nbs3-Rdg2a* differed significantly from the previous two: the coding sequences was 5,397 bp long and was predicted to be organized in four exons of 2,065 bp, 1,209 bp, 471 bp and 1,652 bp, from start codon to stop codon, separated from three introns of 167 bp, 792 bp and 56 bp, respectively. A RACE analysis of the *Nbs3-Rdg2a* mRNAs showed that these putative introns were not spliced and that this gene carries other three introns: one of 305 bp located in the 5' UTR, one 70 bp-long in the 3' UTR and another intron of 44 bp located immediately after the start codon and subjected to alternative splicing. This last intron was very interesting because its splicing causes a frame-shift, resulting in termination after the first 37 aminoacids, while retention leads to termination after the first four and a half LRR units (Bulgarelli e*t al.*, 2010). In both cases, however, it is highly probable that *Nbs3-Rdg2a* encodes for not functional truncated proteins and was therefore excluded as an *Rdg2a* candidate.

Horvath and co-workers (2002) were not able to amplify the barley *Rpg1* gene (a non-NB-LRR gene that confers resistance to rust) sequence on a susceptible variety, using several primer combinations designed on the genomic sequence. In this case, the susceptible cultivar did not carry the *Rpg1* locus. Nevertheless, it is possible that the locus is rearranged in the susceptible genotypes (insertion/deletion; duplication, inversion… could be occured), as observed when resistant and susceptible haplotypes for barley resistance loci *Mla* (Shen *et al.*, 2003) and *Rph7* (Scherrer *et al.*, 2005) were compared. Thus, to asses whether the susceptible cv. Mirco carries the *Rdg2a* locus and to analyze the differences between the resistant and susceptible loci, the two Mirco genes were sequenced using primers designed on *Nbs1-Rdg2a* and *Nbs2-Rdg2a* genomic sequences. Both the genes were present in the susceptible variety, demonstrating that Mirco carries the *Rdg2a* locus. The main differences were in the promoter regions: two insertions of 436 bp (next to a putative TATA-box element) and 854 bp, respectively, for Mirco *Nbs1-rdg2a* and a deletion for *Nbs2-rdg2a*, at the level of a trasponson MITE (*M*iniature *I*nverted *T*ransposable *E*lement)-like element. The *Nbs2-Rdg2a* and *Nbs2-rdg2a* alleles gave 93.1% of identity when compared, apart for the MITE insertion in Thibaut. Interestingly, the PromH programme for prediction of plant promoters (http://www.softberry.ru/berry.phtml?group=programs&subgroup=promoter&topic=tssp; Solovyev and Shahmuradov, 2003) identified potential binding sites for transcription factors, a TATA-box element and a likely promoter whithin the MITE (Bulgarelli *et al.*, 2010). Moreover, this insertion/deletion polymorphism pattern was found to co-segregate with the *Rdg2a* locus in the high resolution mapping population, thus demonstrating that *Nbs2-rdg2a* represents the Mirco allele.

These results prompted the investigation of the expression of the two genes in the susceptible genotype. RT-PCR analyses revealed that *Nbs1-Rdg2a* and *Nbs2-Rdg2a* are expressed in resistant NIL4876 embryo tissues and leaves, both in challenged and unchallenged conditions at 7 and 14 dai, while the susceptible cv. Mirco did not show transcription of the genes (Bulgarelli *et al.*, 2010). Since expression analysis was carried out with a pair of near isogenic lines, in which the *Rdg2a* locus is the only difference, these data evidentiate and confirm that this locus is involved in the resistance response to *P. graminea* isolate *Dg2* but again did not allow the exclusion

of any of the two candidates. A possible explanation for the lack of transcription in Mirco is that the expression of *R* genes imposes high fitness costs in the absence of the disease (Tian *et al.*, 2003), thus unnecessary *R* genes may become rapidily inactivated (Michelmore *et al.*, 1998). The rearramgements in the promoter regions of Mirco genes may be the cause of this inactivation in Mirco. The MITE-like element may have contributed to the functionalization of the Thibaut *Nbs2-Rdg2a* allele, owing to the presence of potential binding sites for transcription factors, like TATA-box element. This effect is similar to that of the *Renovator* retrotransposon insertion for the rice blast resistance gene *Pit*. Indeed, the 3' LTR of *Renovator*, located just upstream the *Pit* gene in the resistant cv. K59, contains a promoter region that activates *Pit* transcription (Hyashi and Yoshida, 2009). These observations are in agreement with first described by McClintock (1956) and others (Chuck *et al.*, 2007; Kashkush e*t al.* 2003; Martienssen *et al.*, 1990; Masson *et al.*, 1987; Errede e*t al.*, 1980), *T*ransposable *E*lements (TEs) have the ability to affect the expression of neighboring genes.

qRT-PCR data revealed that the *Nbs1-Rdg2a* transcription is not pathogen responsive, while the *Nbs2-Rdg2a* transcripts level increased significantly during the first stages of infection and was unresponsive by 22 dai. Neverthless, *Nbs2-Rdg2a* mRNA was 2 to 16 times less abundant than *Nbs1-Rdg2a* transcript, depending on time-point and inoculation treatment (Bulgarelli *et al.*, 2010). It was therefore not possible to trace back the fungal-unresponsiveness of *Nbs1-Rdg2a* transcription to a lack of a role in resistance. Actually, most of the race-specific plant disease resistance genes are constitutively expressed. For example, The *Ag15* gene, located in the *Lr19* locus that confers resistance to leaf rust in wheat, showed no significant variations in transcript level detected between inoculated and mock-inoculated leaves (Gennaro *et al.*, 2009). Even the mRNA level of the flax rust resistance gene *L6* did not vary during incompatible host-pathogen interactions (Ayliffe *et al.*, 1999) and similarly, rice bacterial blight resistance gene *Xa21*, rice blast resistance gene *Pi36* and tomato resistance gene *Pto* were not induced by infection with the incompatible pathogens (Liu *et al.*, 2007; Century *et al.*, 1999; Martin *et al.*, 1993). The constitutive expression of *R* genes is consistent with the need for a rapid

response to pathogen attack to which NB-LRR proteins must respond (McHale *et al.*, 2006; Michelmore, 2000). It should be noted, however, that our experimental protocol would not allow the detection of small or localized changes in the expression of candidates after infection because RNA from whole embryo was analyzed.

Given the absence of indirect evidences complementation experiments of a susceptible genotype were carried out to validate the candidates. The successful barley *Agrobacterium*-mediated transformation is still linked to the variety Golden Promise (Shrawat *et al.*, 2007). Fortunately, it was possible to utilize the Agroinfiltration method because Golden Promise is susceptible to *P. graminea-Dg2*. Golden Promise was transformed with *Nbs1-Rdg2a* and *Nbs2-Rdg2a* genes under control of their native 5' and 3' regulatory sequences and T_1 plants, derived from the self-pollination of transformed T_0 lines, were tested for resistance to isolates *Dg2* and *Dg5*. Transgenic expression of *Nbs1-Rdg2a* conferred a complete resistance to isolate *Dg2* but was ineffective towards isolate *Dg5* (Bulgarelli *et al.*, 2010). Moreover, in presence of the *Nbs1-Rdg2a* transgene the fungal *Ubiquitin* and *GTPase activator* genes were not expressed in leaves, thus indicating that this gene arrested fungal growth at the embryo level, like *Rdg2a* (Haegi *et al.*, 2008). On the other hand, T_1 *Nbs2-Rdg2a* transgenic plants were completely susceptible to both isolates. Based on these observations and by the fact that *Nbs1-Rdg2a* could confer the same resistance specificity as *Rdg2a*, we concluded that *Nbs1-Rdg2a* is the *Rdg2a* gene and most importantly, *Rdg2a* is the first seed-borne resistance genes to be cloned and characterized. Analysis of near-isogenic lines indicated that the *Rdg2a* locus controls partial to strong resistance to at least four others isolates of leaf stripe pathogen (Tab. 1.2, Par. 1.3.1, Introduction; Bulgarelli *et al.*, 2010), therefore transgenic plants homoziguos for *Rdg2a* are being tested with different isolates of *P. graminea* and the same analysis will be performed on transgenic plants expressing this gene constitutively, under control of the maize *Polyubiquitin* promoter (plants are in preparation). These analyses will help to confirm whether really *Nbs1-Rdg2a* gives the same resistance pattern as *Rdg2a*.

At this point the question was: is *Nbs2-Rdg2a* involved in resistance against *Pyrenophora graminea* too? Both *Nbs1-Rdg2a* and *Nbs2-Rdg2a* encode for very similar CC-NB-LRR proteins and showed no differences in the motifs recognised as being conserved across this class of resistance proteins, except for a conservative amino acid substitution in the CC motif. The most significative variations are limited in the LRR domain because NB2-RDG2A presented a deletion of three LRR units and the comparison of the two proteins showed 77.9% of identity for this domain with respect the 83.8% for the NB domain. Variations between R gene alleles or paralogues reported to abolish resistance function include both single amino acid substitutions (Bryan e*t al.*, 2000, Dinesh-Kumar *et al.*, 2000) and the absence or substitution of a section of the LRR domain encompassing one to several repeat units (Feulliet e*t al.*, 2003; Anderson *et al.*, 1997). Feulliet *et al.* (2003) demonstrated that a non-functional mutant for the *Lr10* gene (resistance to *Puccinia triticina* in wheat) did not carry 5 LRR repeats (EMS*lr10*). Similar loss of resistance has been observed in mutants of the flax rust resistance *M* gene lacking 426 bp encoding part of the LRR domain (Anderson *et al.*, 1997). It should therefore be considered as a highly probable hypothesis that the rearrangements in NB2-RDG2A LRR domain may have determined the suppression of its function. Another finding that supports the view that this protein does not take part in resistance is the observation that the *Nbs2-Rdg2a* transcript was 2 to 16 times less abundant that that of *Rdg2a*, depending on time-point and inoculation treatment. This lower expression may contribute to the inactivity of the protein, as observed for the potato *Nbs-LRR* late blight resistance gene *RB* (Kramer *et al.*, 2009) and rice receptor kinase-like bacterial blight resistance gene *Xa3* (Cao *et al.*, 2007), whose transcript aboundance correlates with their resistance activity. To investigate this possibility, cv. Golden Promise will be transformed with *Nbs2-Rdg2a* under the control of the maize *Polyubiquitin* promoter, to overexpress the gene. However, since *Nbs2-Rdg2a* contains a complete open reading frame and is expressed in embryo tissues, we could not exclude a role in resistance to *P. graminea*. In order to detect whether *Nbs2-Rdg2a* is affective against the fungus, transgenic plants homozygous for this gene will be screened for resistance to different isolates.

In addition, the idea that the adaptive evolution of LRR domains allows for a rapid generation of altered recognition specificities has been confirmed by much evidences (Lehman, 2002). In particular, the highest rates of amino acids replacement changes are shown in the solvent-exposed residues of the LRR domain (Stahl and Bishop, 2000). This is consistent with the fact that this domain governs race-specific pathogen recognition and an adaptive evolution is in agreement with an evolutionary arm race in this respect that pathogens should impose selection to continually alter recognition specificity (Lehman, 2002). Moreoever, the modification of the number of the LRR repeats, mainly due to recombination followed by gene conversion, appears to be an important contributor to *R* gene diversification because the reduction/expansion events could change the spatial distribution of ligand contact point and adjust either affinity or specificity for different ligands (Ellis *et al.*, 2000). Considering these observations, we could not exclude that *Nbs2-Rdg2a* plays a role in resistance to *Pyrenophora graminea* just because it shows the deletion of three LRR repeats.

4.1.2 Evolution at the *Rdg2a* locus

Rdg2a resides in a gene cluster, as does many other NB-LRR-encoding genes. (Meyers *et al.*, 2003). This organization might facilitate sequence exchanges between paralogues and generating new resistance specificities (Mondragon-Palomino *et al.*, 2002; Kuang *et al.*, 2004), as well as expansion and contraction of gene copy number in the gene family (Leister, 2004). At the *Rdg2a* locus, paralogs appear to be the result of relatively recent gene duplications as demonstrated by the strong DNA sequence identity between the three family members that, in the case of *Nbs2-Rdg2a* and *Nbs3-Rdg2a*, extends into the 5' untrascribed region (Bulgarelli *et al.*, 2010). This is consistent with the observation that *R* genes, in particular NB-LRR-encoding genes, have high-levels of inter- and intraspecific variation and also high rates of mutation and recombination (McHale *et al.*, 2006; Kuang *et al.*, 2004). Molecular analysis of the *Cf-2/Cf-5* and *Cf-4/Cf-9* loci demonstrated that unequal crossing-over and/or gene conversion have played a fundamental role in their evolution (Parniske e*t al.*, 1997). The presence of two similar copies of *Cf-2* is most likely the result of a recent sequence duplication, due probably to an unequal

crossing-over (Dixon e*t al.*, 1996). Also *Cf-9* and *Cf-4* differ by one nucleotide at their 3'ends and are identical for a further 5.3 kb downstream (Thomas e*t al.*, 1997). The noted variable sequence patches could be generated either by successive rounds of reciprocal recombination or by gene conversion events (Lehmann, 2002). The unusual structure of *Nbs3-Rdg2a,* in which sequences encoding part of the NB and LRR regions are duplicated, together with the deletion of the region containing three complete LRR units in NB2-RDG2A, provide further examples of variation in the *Rdg2a* locus generated by recombination. Several examples are available in which diversifying selection contributes to sequence diversity at *R* gene loci (Ellis *et al.*, 2000), but they are limited for *R* genes that encode receptors that directly interact with pathogen effectors. In the case of the *Cf9* locus of *Cladosporium fulvum* resistance in *Lycopersicum* species, a functional *9DC* gene was found in *Lycopersicum pimpinellifolium* resulting from an unequal crossing-over between the *Cf9* resistance gene and its paralogue, the *9D* gene (Kruijt *et al.*, 2004). Recombination between *R* alleles has been described in the flax rust-resistance *L* alleles with a mosaic pattern of conserved sequences among alleles (Ellis *et al.*, 1999). Moreover, Kuang *et al.* (2004) proposed also gene conversion as one of the major mechanism of *R* genes evolution. In contrast to the barley *Mla* alleles, which were shown to evolve by accumulation of small in/dels and point mutations (Halterman and Wise, 2004; Wei *et al.*, 2002), the wheat *Pm3* alleles and their up- and downstream regions evolved either by gene conversion/recombination or by single point mutations (Wicker *et al.*, 2007; Yahiaoui *et al.*, 2006). The comparison of the genomic sequences of *RPW8* loci in three species (*Arabidopsis thaliana*, *Arabidopsis lyrata* and *Brassica rapa*) revealed that *RPW8* has evolved from recent gene duplication and subsequent functional diversification favoured by diversifying selection (Xiao *et al.*, 2004). In contrast to these observations, *R* gene encoding R proteins that detect the presence of the pathogen effectors without a direct contact are conserved through evolution (Bent and Mackey, 2007; Dangl and McDowell, 2006). This is the case of the *Arabidopsis Rpm1*, *Rps2* and *Rps5* loci that act by indirect guard mechanisms and are characterized by low levels of genetic diversity and the presence of ancient polymorphisms. This suggests that simple balanced polymorphisms for functional and non-functional alleles have been maintained over

long evolutionary time scales and the proteins that act by an indirect guard mechanism are under conservative selection (Van der Hoorn *et al.*, 2002; Bergelson e*t al.*, 2001; Stahl *et al.*, 1999).

The fact that the *Rdg2a* is subjected to diversifying selection is consinstent with a coevolutionary arms race between *R* and the corresponding *Avr* genes (Bergelson e*t al.*, 2001; Stahl and Bishop, 2000). According to this model, it may be possible that small conformational changes in the RDG2A protein restore the interaction with variant version of the avirulence gene product that would also be under diversifying selection, similar to what Dodds *et al.* (2004) observed for the flax rust *AvrL567* gene and to what happens for the downy mildew ATR1^{NdWsB} protein, recognized by the *Arabidopsis* RRP1 protein (Rehmany *et al.*, 2005). Both genes, indeed, are characterized by strong positive selection for amino acid variation.

Diversifying selection at the *Rdg2a* locus is also supported by the observation that in two leaf stripe susceptible genotypes analyzed, Mirco and Morex, *Rdg2a* homolog sequences are present in syntenic positions. In particular, in cv. Morex, sequences sharing more than 93% of identity to *Rdg2a* were identified both in coding and non-coding regions and deletions of intergenic regions and of members of the gene family are most likely responsible for the rearrangements suggested by Sounthern blot analyses (Fig. 1.11, Par. 1.3.1, Introduction). It is possible that unequal crossing-overs generated deletions in Morex producing a hybrid gene between *Rdg2a* and *Nbs2-Rdg2a* ancestors. Whether this gene has a function against any isolate of *Pyrenophora graminea* is under investigation. However, the lack of a complete allele of *Nbs1-Rdg2a* in this *Dg2*-susceptible genotype further proves that *Nbs1-Rdg2a* is the *Rdg2a* gene. The sequencing of the Morex *Nbs3-Rdg2a* allele is currently in progress.

4.1.3 Haplotype analysis at the *Rdg2a* locus

PCR-based molecular marker analyses of different barley varieties showed that Thibaut *Rdg2a* locus is largely conserved in both *Dg2*-resistant and susceptible plants. Main differences are limited in the region of the *Pro* locus, encoding for a protease, that seems to be re-arranged, with respect to Thibaut, in most genotypes. Moreover, for a few varieties, a small deletion is also present at the level of the

Rdg2a promoter. The tests performed at five loci within the *Rdg2a* locus identified four different haplotypes. Interestinlgy, four genotypes resistant to isolate *Dg2* but susceptible to isolate *Dg5* (Optic, Galleon, Haruna Nijo and Acuario) showed Thibaut haplotype with the exclusion of a slightly shorter amplification yielded by the primer combination CR3 *plus* NCR2. Also Rebelle genotype demonstrated to belong to this haplotype group. The sequencing of the *Rdg2a* gene in three *Dg2*-resistant cultivars (Rebelle, Galleon and Haruna Nijo) was carried out and revealed a strong sequence conservation that led to the synthesis of very similar proteins. In the LRR domain of Galleon RDG2A there are only four amino acid substitutions that are located in the fourth and the fourtheenth LRR units but do not belong to the β-strand/β-turn. Moreover, Haruna Nijo RDG2A shows two amino acids changes between the CC and NB domains. As the two varieties are fully resistant to isolate *Dg2*, these changes may have not influenced the RDG2A function. One possible explanation is that the amino acids that result from the nucleotide substitutions have the same chemical characteristic of those present in Thibaut RDG2A. By the fact that the CC, NB and LRR domains are the most important for protein activity, it is also plausible that the substitutions occurred in positions that do not have any functional role in the protein; also, the observed changes in Galleon could indicate that this allele was subjected to diversifying selection. The cloning of *Rdg2a* is therefore facilitating allele sequencing from different barley genotypes (allele mining) and expression analyses of homologues from both wild and cultivated barley (Bulgarelli *et al.*, 2010). A similar approach was carried out for functional *Pm3* alleles from both wild tetraploid and landraces of bread wheat, allowing a significant expansion of wheat *R* genes available against powdery mildew (Bhullar *et al.*, 2009; Yahiaoui *et al.*, 2009). It would be interesting to performe an high-throughput allele mining analysis using the Genome Capture Sure Select Target Enrichment System (Agilent; Gnirke *et al.*, 2009) associated with the *N*ext *G*eneration *S*equencing (NGS), to compare *Rdg2a* alleles in different (wild and cultivated) genetic backgrounds; the approach would allow the identification of new alleles, perhaps with different resistance specificities, that could be deployed in the barley resistance breeding. Another important consideration is that Rebelle, Galleon and Haruna Nijo come from far area of the world and the deep conservation in

Rdg2a sequence strongly demonstrates that this gene is widespread and conserved by both in six-rowed (Thibaut and Rebelle) and two-rowed (Haruna Nijo and Galleon) genotypes.

4.2 RDG2A localizes in nucleus and cytoplasm and confers resistance without programmed cell death

4.2.1 Sub-cellular localization of the RDG2A protein

Fluorescence from transientely expressed RDG2A-YFP fusion protein was abundant in the nucleus, despite the fact that it lacks of a NLS motif, and was also present in the cytoplasm, suggesting that resistance functions of RDG2A might relate to one or both of these locations. In barley, intracellular mildew A (MLA) R proteins function in the nucleus to confer resistance against the powdery mildew fungus. After the recognition of the fungal avirulence A10 effector by MLA10, this protein induces nuclear associations between receptor and the repressor of PAMP-triggered basal defense WRKY1 transcription factor, leading to a de-repression of basal defence mechanisms and effective immunity (Shen *et al.*, 2007). *WRKY38*, a *WRKY1* allele, is up-regulated upon *P. graminea* isolate *Dg2* infection (Haegi e*t al.*, 2008). It would be interesting to investigate whether RDG2A interacts with this transcription factor and whether this interaction has a role in resistance to the fungus. However, it should be noted that RDG2A localization was determined in leaves of uninfected plants, and that the location of resistance protein might be different in embryo tissues and during the infection (Bulgarelli *et al.*, 2010). Localisation studies have showed, in fact, that NB-LRR proteins were found to be localized in a variety of sub-cellular compartements (Rafiqi e*t al.*, 2009). Nevertheless, nuclear localisation has so far been shown only for a few plant NB-LRR proteins and many cloned *R* genes, like RDG2A, do not carry an obvious NLS motif. The large size of these proteins would preclude passive diffusion through the nuclear pores complexes, although interaction with other cellular proteins capable of actively entering the nucleus could results in nuclear localization (Rafiqi *et al.*, 2009). For example, the *Arabidopsis* TIR-NB-LRR RRS1-R protein nuclear localization depends on the

presence of the bacterial PopP2 effector, which carries a functional NLS (Mangelsen *et al.*, 2008).

To mine the mechanism of action of RDG2A we should answer to several questions: how can the avirulence factor enter into contact with the cytoplasmatic RDG2A? Why should the pathogen "send" an Avr factor inside the plant cell and risk being detected? What happens after the recognition of the avirulence factors?

It has been suggested that *R* genes directely or indirectely recognize the Avr factors of pathogens (Dangl and Jones, 2001). Galli e*t al.* (2010) hypothesized that the recognition of the Avr factor by the RVI15(VR2) protein, confering resistance against apple scab and located in the cytosol, can acts in two ways: recognizing a product from the cuticle degradation, which passes the cell wall; or recognizing a product from the degradation of the cell wall observed after infection at the point of pathogen penetration. In the case of *Pyrenophora graminea*, the fungus grows only intercellurly, whitouth forming austoria (Haegi *et al.*, 2008; Platenkamp, 1976) and whithout penetrating the cell wall, thus it is likely that the avirulance factors are transported into the host cell across the plasma membrane and RDG2A recognizes them inside the host cell (Bulgarelli *et al.*, 2010). Prediction analyses of protein domains did not reveal any extracellular LRR repeats for RDG2A, but an interaction between this protein and a transmembrane receptor can not be excluded.

4.2.2 RDG2A confers resistance in the absence of programmed cell death

Most of the *R* genes function involves the induction of a *H*ypersensible *R*esponce (HR) through *P*rogrammed *C*ell *D*eath (PCD) restricted to infected cells (Jones, 2001). For example, the *Arabidopsis RPS4* gene belongs to the *T*oll/interleukin-1 receptor/*N*ucleotide-*B*inding site/*L*eucine-*R*ich *R*epeat (TIR-NB-LRR) class of plant resistance genes and confers resistance to *Pseudomonas syringae* triggering HR in leaves through its TIR domain (Swiderski *et al.*, 2009; Zhang *et al.*, 2004). The Cyst Nematode SPRYSEC protein RBP-1 triggers cell death in *Arabidopsis* by eliciting Gpa2 and RanGAP2 NB-LRR proteins (Sacco *et al.*, 2009). To find out whether also *Rdg2a* induces HR, a TUNEL (*T*erminal deoxynucleotidyl transferase d*U*TP *N*ick and *L*abelled) analysis was carried out on longitudinal sections of infected and

control embryos of NIL3876 at three different time-points (14, 22 and 26 dai). The TUNEL method enables detection of free 3'-OH groups generating by DNA breaks that occur at the first stage of Programmed Cell Death. The use of this technique in plants is largely reported (Demidchik *et al.*, 2010; Koroleva *et al.*, 2010; Ma *et al.*, 2010; Serrano *et al.*, 2010; Souza *et al.*, 2010; Casani *et al.*, 2009; Bozhkov *et al.*, 2005; Coffen and Wolpert, 2004; Deuschle *et al.*, 2004; Dominguez *et al.*, 2002; Brodersen *et al.*, 2002; Balk and Leaver, 2001; Dickman *et al.*, 2001; Fath *et al.*, 2001; 1999; Schopfer *et al.*, 2001; Asai *et al.*, 2000; Gomès *et al.*, 2000; Koch *et al.*, 2000; Sasabe *et al.*, 2000; Xu and Roossinck *et al.*, 2000; Groover and Jones, 1999; Schmid *et al.*, 1999; Tamagnone *et al.*, 1998), but there are only two examples of TUNEL reaction conducted on longitudinal sections of embryos (Giuliani *et al.*, 2002; Fath *et al.*, 2000).

Interestingly, no significative TUNEL differences between infected and controls embryos were found at the junction of the scutellum and scutellar node, where resistance against *P. graminea* takes place (Haegi *et al.*, 2008). Furthermore, in these tissues the number of cells showing cell death was transcurable, even where the presence of the fungus was identified by Calcofluor staining. This finding was also supported by the fact that no necrotic tissues or cell collapse were observed under bright views for the embryo regions showing autofluorescence, a marker for infection (Haegi *et al.*, 2008). Autofluorescence, in fact, was detected at the junction of the scutellum and scutellar node tissues in the resistance response to leaf stripe, but was confined to the cell walls (Bulgarelli *et al.*, 2010; Haegi *et al.*, 2008). Taken together, these observations demonstrate that *Rdg2a* induces resistance withouth triggering PCD. There are few other examples of NB-LRR mediated resistance that does not involve Programmed Cell Death. The barley *Mla1* powdery mildew resistance gene does not induce HR (Bieri *et al.*, 2004), although the *Mla12* allele exhibits necrotic reactions (Freialdenhoven *et al.*, 1994). Even the *Arabidopsis RPS4* and *RPS6* genes confer bacterial resistance in a HR-indipendent manner (Gassman *et al.*, 2005). The same happens for the *Rx* gene in potato and it has been proposed that the lack of HR is probably due to the fact that the resistance mechanism is so rapid that prevents the accumulation of the avirulance factor to levels that would otherwise trigger a more extensive host response (Bendahmane *et al.*, 1999). In our

case, HR-associated resistance response would be too damaging to the embryo, and therefore unviable evolutionarly. In addition, HR deprives obligate pathogens of living host cells required for successfull colonization, but may be favourable to the hemibiotrophic *Pyrenophora graminea*, which obtains nutrients by hydrolytic degradation of host cell walls (Bulgarelli e*t al.*, 2010).

Thus, how does the *Rdg2a* resistance take place? It has been observed that *Rdg2a* resistance is associated with accumulation of compounds (most likely phenolic compounds) determing cell wall localized autofluorescence at the scutellar node and at the basal regions of provascular tissues in infected embryos (Bulgarelli *et al.*, 2010; Haegi e*t al.*, 2008). Heagi and co-workers (2008) performed microarray analyses and a pathogen-induced up-regulation for several genes related to cell wall modification was observed in the resistant NIL3876 but not in the susceptible Mirco. Among these genes they identified those coding for xylose isomerase and arabinoxylan arabinofuranohydrolase (AXAH) that are involved in the hydrolysis of the complex heteroxylan polysaccharides of the primary cell wall. Furthermore, *Callose Synthase* and *Peroxidase* genes were induced in infected NIL3876 only. Callose forms a major component of papillae deposited on the inner face of the cell wall in response to pathogen challenge (Schulze-Lefert, 2004) and peroxidases are involved in lignifications and cross-linking of phenolics, proteins and carbohydrates (Moerchbacher, 1992). Also potentially related to cell-wall reinforcement is the production of ROS (*R*eactive *O*xygen *S*pecies). The authors found that genes encoding H_2O_2-generating enzymes (germin F and oxalate oxidase) are induced at a high level in the resistant NIL3876-*Dg2*. These enzymes are both located in the cell wall (Cona *et al.*, 2006; Zhou *et al.*, 1998) and can contribute to a local generation of H_2O_2 for both cross-linking of cell-wall components and defense signalling. Consisting with a higher production of H_2O_2 in infected NIL3876 some genes involved in ROS detoxification protection are expressed at higher level with respect to susceptible and non-infected resistant plants. Belonging to this class of genes is the ascorbate peroxidase and cyclopropane fatty acid synthase genes whose products uses S-adenosylmethionine to generate a methylene bridge across the double bonds in unsaturated fatty acids, contributing to the protection of membranes and other cellular components from damage by ROS (Anthony *et al.*, 2005). Hence, *Rdg2a*

probably mediates resistance to leaf stripe inducing secretory immune responses, leading to physical and chemical barriers to infection in the cell walls and intercellular spaces of embryo tissues, without triggering Programmed Cell Death.

5. Conclusions and perspectives

In this study we were able to clone and characterize the first seed-borne disease resistance gene: the *Rdg2a* gene that confers resistance to the hemi-biotrophic fungus *Pyrenophora graminea* (the causal agent of barley leaf stripe) isolate *Dg2*. In a previous analysis, a map-based molecular cloning of the *Rdg2a* locus identified three putative homologous CC-NB-LRR encoding genes: two intronless, *Nbs1-Rdg2a* and *Nbs2-Rdg2a* and one carrying five introns, *Nbs3-Rdg2a*. RACE analyses revealed that four of these introns were unprocessed and one was subjected to alternative splicing, leading to the productions of truncated proteins. Thus, *Nbs3-Rdg2a* was excluded from acting in the resistance against isolate *Dg2*.

Neither *Nbs1-Rdg2a* nor *Nbs2-Rdg2a* are expressed in the susceptible cv. Mirco, but are transcribed in the embryo and leaf tissues of its resistant *N*ear *I*sogenic *L*ine, NIL3876. Rearrangements in the promoter region caused by insertion/deletion of transposable elements may explain the lack of expression of these genes in Mirco. Complementation analyses using the two genes independently, under the control of their native promoter, revealed that only *Nbs1-Rdg2a* leads to the rescue of resistance, suggesting that this gene is the *Rdg2a* gene.

By the fact that *Nbs2-Rdg2a* encodes for a protein lacking of three leucine rich repeats, with respect to RDG2A, and that is significatively less transcribed than *Rdg2a*, we could think that it is not involved in *Dg2*-resistance. However, as it contains a complete open reading frame and is expressed in resistant embryos, it might be involve in resistance against other *P. graminea* isolates.

Moreover, transgenic plants homozygous for *Nbs1-Rdg2a* and *Nbs2-Rdg2a* are under investigation for resistance to different isolates of the fungus to confirm if the first gene confers the same resistance pattern of *Rdg2a* and whether *Nbs2-Rdg2a* is effective against any other isolate. To better validate our findings and to identify whether there is a correlation between *Nbs2-Rdg2a* transcription level and its activity, we will performe phenotypic test, using several *P. graminea* isolates, on transgenic plants overexpressing the two genes, under the control of the maize *Poly-Ubiquitin* promoter region.

Rdg2a resides in a gene cluster, as does many other resistance genes and this organization can promote unequal recombination, resulting in sequence exchange between paralogs and generation of recombinant genes with new resistance specificities, as well as expansion/contraction of gene copy number. Cv. Morex, for example, carries two deletions that reduced the number of genes to two and generated a hybrid gene between *Rdg2* and *Nbs2-Rdg2a*. The strong DNA sequence identity of the three genes at the *Rdg2a* locus, demonstrates that they are the result of a recent gene duplication. This locus is also subject to diversifying selection, consinsting with the model in which *R* genes co-evolve with pathogen effectors, due to direct interaction of the gene products. Importantly, *Rdg2a* is highly conserved in different genetic backgrounds coming from far areas of the world, underling the importance and the wide spread of this gene.

The majoirity of resistance genes induce programmed cell death at the lvevel of infected cells for contrasting pathogens. *Rdg2a*, like a small number of *R* genes, is an exception because it does not triggers a hypersensitive responce to the fungus; it activates instead the expression of genes involved in the strengthening of physical and chemical barriers to infection in the cell walls and intercellular spaces of the embryo tissues.

Several questions about the mechanisms through which RDG2A triggers the resistance responses need to be answered: what are the immediate downstream targets that this protein modulates in order to activate the defense response?

What are the pathogen effectors and the host processes that effectors target?

How can knowledge of elicitors, effectors and *R* genes be translated into practical disease control measures that confer durable disease resistance?

It is widely demonstrated that the functionality of R protein is associated to chaperone complexes and conformational changes upon Avr detection that initiates the signal transductions. It would be interesting to check, by protein-protein interaction assays, which proteins are also involved in the *Rdg2a*-mediated resistance.

In molecular studies of plant-microbe interactions most of the work is carried out on the host, while little is understood about the pathogen, especially for fungi (Jones and Dangl; 2006). Thus, the identification of the AvrRDG2A and also of the host

processes that are corrupted by this protein and where this protein is secreted and/or delivered would be done. Moreover, the possibility to perform a stable transformation of *P. graminea* will give the opportunity to test *in vivo* any putative gene involved in disease establishment/pathogen recognition.

To date, only Thibaut *Rdg2a* and Vada *Rdg1a* resistant alleles are used in breeding and provides useful resistance against leaf stripe, but they are not effective towards all isolates (Biselli *et al.*, 2010; Bulgarelli *et al.*, 2010; Gatti *et al.*, 1992). The completely elucidation of the whole barley-*P. graminea* interactions could sustain the crop improvement by taking in consideration the main factors (*e.g.* genes encoding for downstream signalling molecules, transcription factors; regulatory sequences/promoter of genes up-regulated in disease resistance, pathogen effectors…) that act in this pathosysitem in order to obtain new practical applications useful to contrast other *P. graminea* isolates or other seed-borne diseases. Furthermore, the new rapid and cheap methods of sequencing based on the *N*ext *G*eneration *S*equencing (NGS) represent a powerfull tool to extensively analyze the genomes of crop species. In this view, the *Rdg2a* sequence could be used as probe in allele mining experiments, through genome capture associated to NGS, on wild and cultivated barley varieties to better understand the mechanisms at the base of the evolution of the *Rdg2a* locus and, mainly, to identify further alleles with different specificity to *P. graminea* isolates. Thus, it will be possible to amplify the range of resistance genes available to breeders and better contrast the spread of virulent isolates.

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Supplementary materials

Morex mwg7_HVVMRXALLhA425O23_c2 contig (28037 bp long)

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The 1814 bp long fragment is in cursive.

Rebelle *Rdg2a*

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Galleon *Rdg2a*

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Haruna Nijo *Rdg2a*

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Rebelle RDG2A hypothetical protein

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Galleon RDG2A hypothetical protein

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Haruna Nijo RDG2A hypothetical protein

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Publications

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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* (*R*esistance to *D*rechslera *g*raminea) 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*-gene is effective. Histological analysis demonstrated that *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.

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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 parenchymatic 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 coleorhizae, 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* (*R*esistance to *D*rechslera *g*raminea) 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.*

vulgare, 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 F_1 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 146.9-5-6) 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 146.9-5-6 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

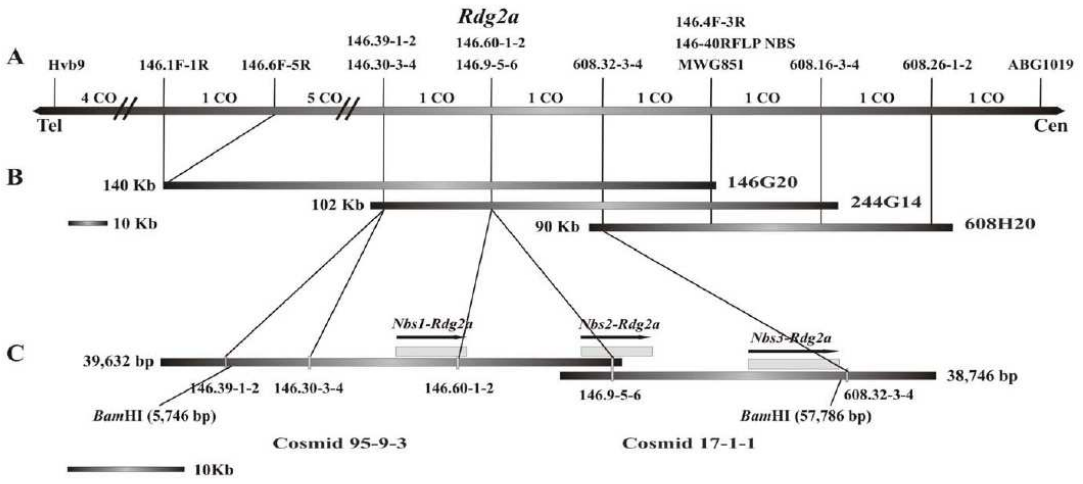

Figure 1. Genetic and physical maps of the *Rdg2a* locus. (A) Genetic map of *Rdg2a*. Crossovers identified in the 1,400 F_2 plants from a cross between Thibaut (*Rdg2a*) and Mirco [7] are shown at the top (CO). Orientation is indicated by Tel (telomere) and Cen (centromere). (B) Contig of Morex BAC clones. (C) Thibaut cosmid contig and genes at the *Rdg2a* locus. Transcription direction of the genes are indicated by arrows.
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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 *Bam*HI 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 NIL3876 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 (cvs. 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 305 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 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′ untranscribed 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/ITMI/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′-untranscribed 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 NIL3876-*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 NIL3876-*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 NIL3876-*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 T_0 lines for each transgene were allowed to self-pollinate and the resulting T_1 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 T_1 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). T_1 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 Dg5 (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*.

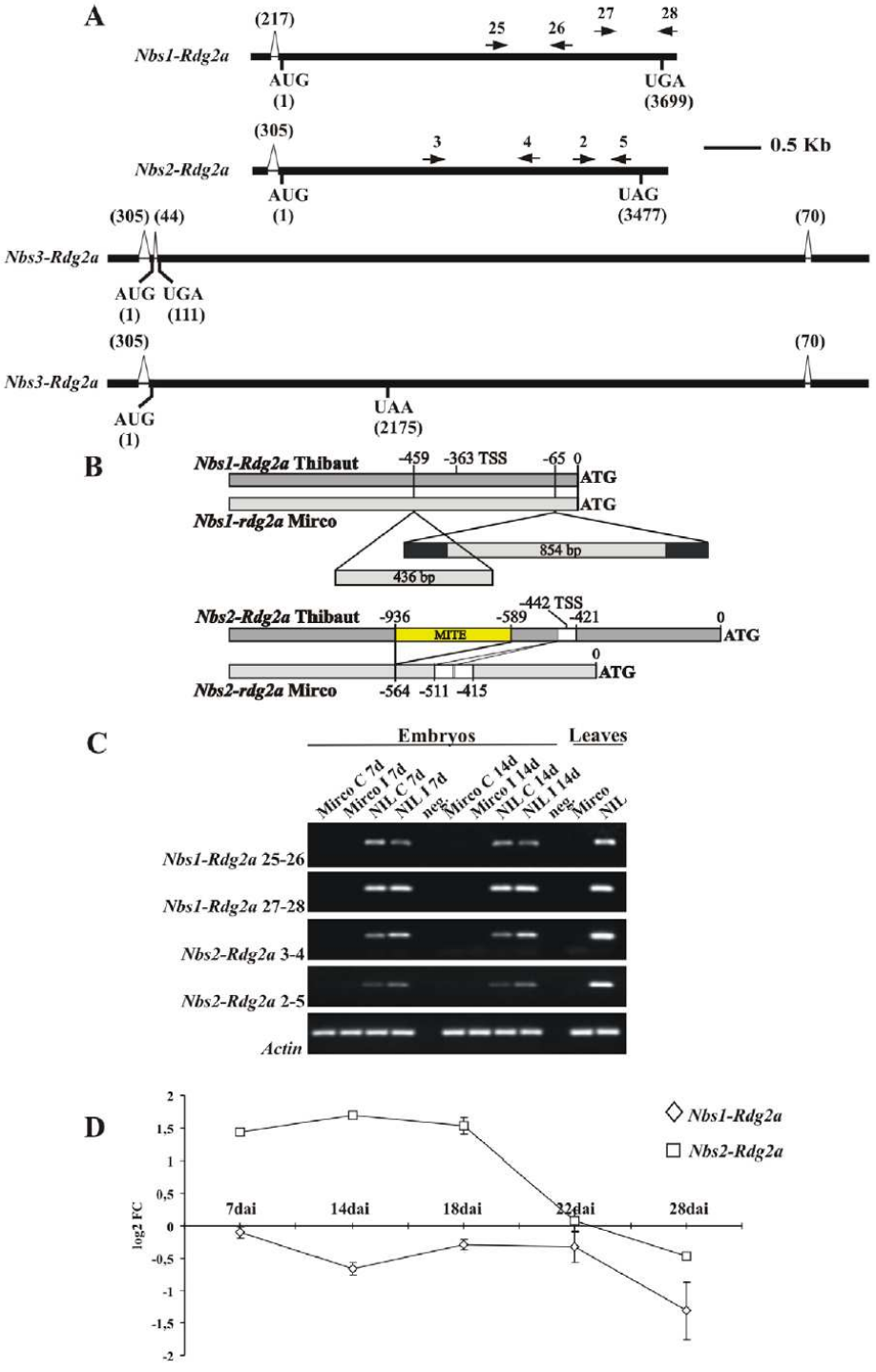
A
(217)
25
26
27
28
Nbs1-Rdg2a
AUG
(1)
UGA
(3699)
(305)
3
4
2
5
0.5 Kb
Nbs2-Rdg2a
AUG
(1)
UAG
(3477)
(305) (44)
(70)
Nbs3-Rdg2a
AUG UGA
(1) (111)
(305)
(70)
Nbs3-Rdg2a
AUG
(1)
UAA
(2175)
B
Nbs1-Rdg2a Thibaut
-459 -363 TSS -65 0
ATG
ATG
Nbs1-rdg2a Mirco
854 bp
436 bp
Nbs2-Rdg2a Thibaut
-936
-589
-442 TSS
-421
0
ATG
MITE
0
ATG
Nbs2-rdg2a Mirco
-564 -511 -415
C
Embryos
Leaves
Mirco C 7d
Mirco I 7d
NIL C 7d
NIL I 7d
neg.
Mirco C 14d
Mirco I 14d
NIL C 14d
NIL I 14d
neg.
Mirco
NIL
Nbs1-Rdg2a 25-26
Nbs1-Rdg2a 27-28
Nbs2-Rdg2a 3-4
Nbs2-Rdg2a 2-5
Actin
D
Nbs1-Rdg2a
Nbs2-Rdg2a
log2 FC
2
1,5
1
0,5
0
-0,5
-1
-1,5
-2
7dai
14dai
18dai
22dai
28dai

Figure 2. Analysis of *Rdg2a*-candidate gene transcript structure and regulation. (A) *Nbs1-Rdg2a*, *Nbs2-Rdg2a* and *Nbs3-Rdg2a* transcript structures (cv. Thibaut), indicating positions of primers used in transcript quantification. The two transcript types resulting from alternative splicing pattern of *Nbs3-Rdg2a* are indicated. (B) Structural differences between Thibaut and Mirco alleles of *Nbs1-rdg2a* and *Nbs2-rdg2a* genes in 5′ regions. Positions of insertion/deletions relative to the start codon are shown. Filled sections indicate inverted repeats present in an insertion in the Mirco *Nbs1-rdg2a* gene. The *Nbs2-rdg2a* allele comparison illustrates variation for a MITE insertion and a 41–bp direct repeat (open sections). Transcription start sites (TSS) for *Nbs1-Rdg2a* and *Nbs2-Rdg2a* are indicated. (C) Semi-quantitative RT-PCR analysis of the *Rdg2a*-candidate gene expression using gene specific primers. Transcripts were analysed in embryos of the cv. Mirco (*rdg2a*) and NIL3876 (*Rdg2a*) genotypes at two timepoints, after inoculation with *P. graminea* Dg2 (I), or in uninoculated controls (C). Leaves of uninoculated plants were also analysed. Negative controls (neg.) in which DNA was omitted are included. Primers for cv. Thibaut genes were those represented in (A), while primers for amplifying homologous fragments from cv. Mirco were based on the cv. Mirco gene sequences and positioned within 30 bp of the corresponding Thibaut primers. RT-PCR of the barley β-*actin* gene was used as an internal control. (D) Quantitative RT-PCR at 7, 14, 18, 22 and 28 days after pathogen inoculation (dai) for the two *Rdg2a*-candidates in embryos of NIL3876-*Rdg2a*. Values are expressed as log2 fold changes of transcript levels in the inoculated samples with respect to the transcript levels in un-inoculated barley embryos. Error bars represent SD across all RT-PCR replicates (four to six from each of two independent inoculations).
doi:10.1371/journal.pone.0012599.g002

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].

Figure 5 illustrates similarities between RDG2A and the most similar sequences in the National Center for Biotechnology Information (NCBI) database. RDG2A was most similar (47–52%) over its whole length to five rice disease resistance-like

Table 1. Analysis of transgenic plants.

		Isolate Dg2		Isolate Dg5	
Constructs/barley cvs.	**Lines[a]**	**No. plants[a]**	**No. res. plants[b]**	**No. plants**	**No. res. plants**
Nbs1-Rdg2a	1/S1-T6	19	19	15	0
	4/S1-T6	21	21	13	0
	7/S1-T6	24	24	11	0
	8/S1-T6	23	22	5	0
	16/S1-T6	19	19	12	0
	17/S1-T6	15	14	8	0
	19/S1-T6	7	7	9	0
	25/S1-T6	19	19	12	0
	31/S1-T6	13	13	5	0
	32/S1-T6	19	18	14	0
Nbs2-Rdg2a	41/S1-T7	23	1	17	0
	42/S1-T7	19	1	16	0
	46/S1-T7	16	0	9	0
	54/S1-T7	21	0	4	0
	56/S1-T7	17	1	5	0
	57/S1-T7	26	0	12	0
	60/S1-T7	20	2	16	0
	62/S1-T7	16	0	18	0
	64/S1-T7	17	0	7	0
	71/S1-T7	24	0	16	0
Thibaut (*Rdg2a*)		40[c]	38	6	0
NIL3876 (*Rdg2a*)		35	34	25	0
Mirco (*rdg2a*)		35	0	19	0
Golden Promise (*rdg2a*)		35	2	9	0
15/S1-T6 (empty vector)		36	1	15	0

[a]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.
[b]Number of transgenic T_1 plants without leaf stripe symptoms. Data were pooled from three independent experiments each comprising 5 or more plants per line.
[c]Total number of plants tested as controls.
doi:10.1371/journal.pone.0012599.t001

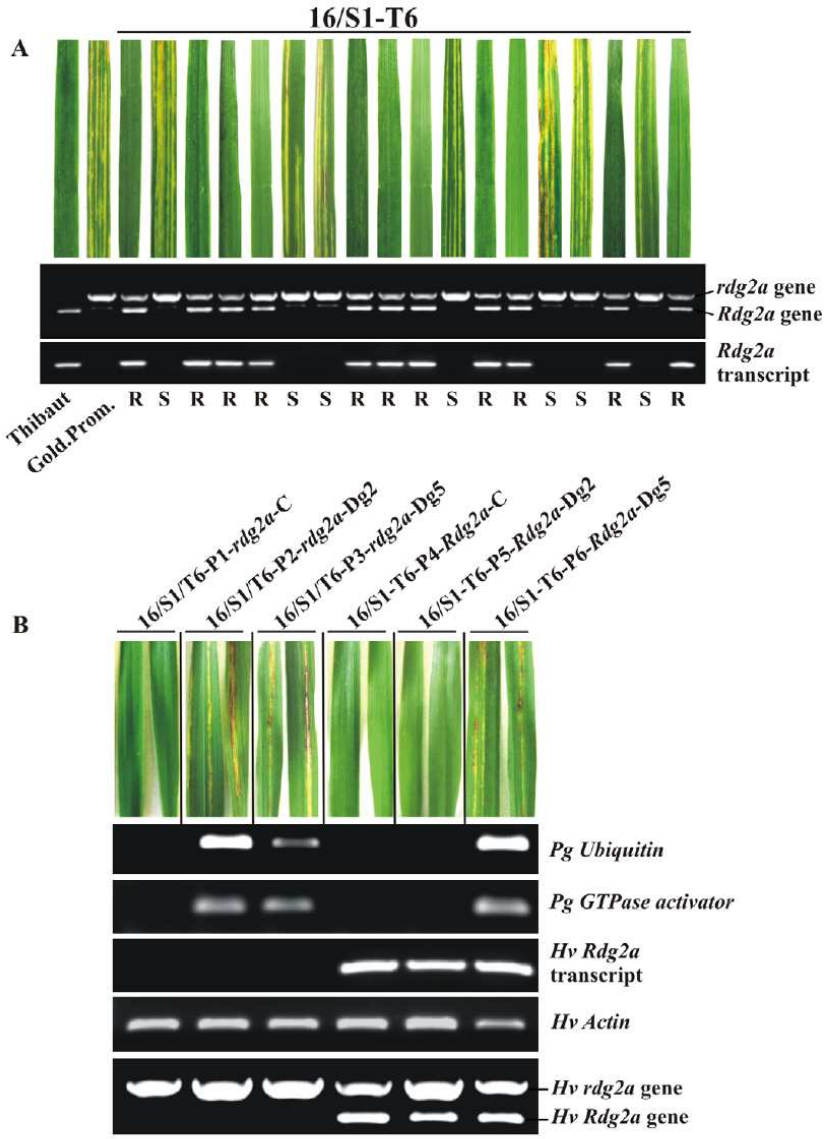

Figure 3. Analysis of T_1 family 16/S1-T6 segregating for the *Nbs1-Rdg2a* transgene. (A) T_1 seeds were inoculated with *P. graminea* isolate Dg2 and plants analyzed for disease symptoms in leaves (upper panel), an STS marker for *Rdg2a* (middle panel; upper band represents the *rdg2a* susceptibility 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 T_1 plants were analysed 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

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 4. RDG2A protein sequence. The predicted coiled-coil (CC) domain is underlined. Motifs conserved in the NB region of NB-LRR proteins are in blue, and are (in order): P-loop, RNBS-A, Kinase 2, RNBS-C, GLPL, RNBS-D and MHD. Amino acids conforming to the cytoplasmic LRR consensus LxxLxLxx(C/N/T)xxLxxLxxLP are in red. CT denotes the RDG2A C-terminal region.
doi:10.1371/journal.pone.0012599.g004

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 (*Ka*) to synonymous (*Ks*) 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 β-strand/β-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

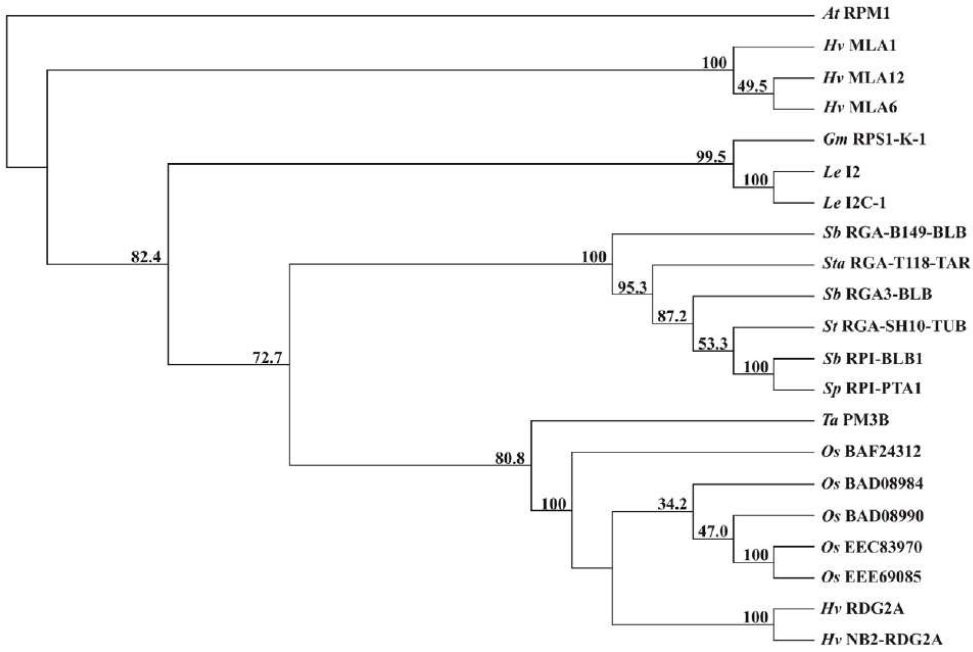

Figure 5. Neighbor-joining phylogenetic tree including RDG2A and similar resistance proteins and resistance gene analog products. Numbers on the branches indicate bootstrap percentages. Prefixes indicate species origin. The *A. thaliana* RPM1 protein (Q39214) was used as an outgroup. Shown are the rice (*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

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

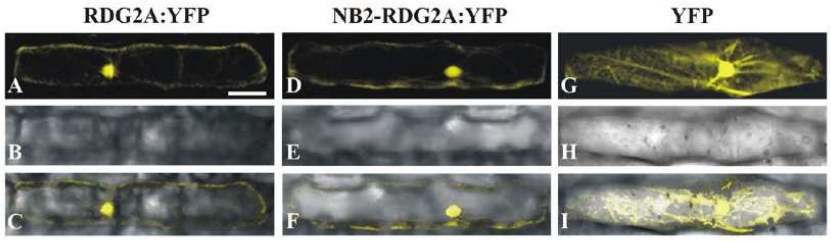

Figure 6. Sub-cellular localization of RDG2A and NB2-RDG2A proteins. Barley cv. Golden Promise epidermal cells were transiently transformed with constructs expressing RDG2A:YFP and NB2-RDG2A:YFP fusion proteins (A and D respectively), driven by the maize *polyubiquitin* gene promoter. A construct expressing 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

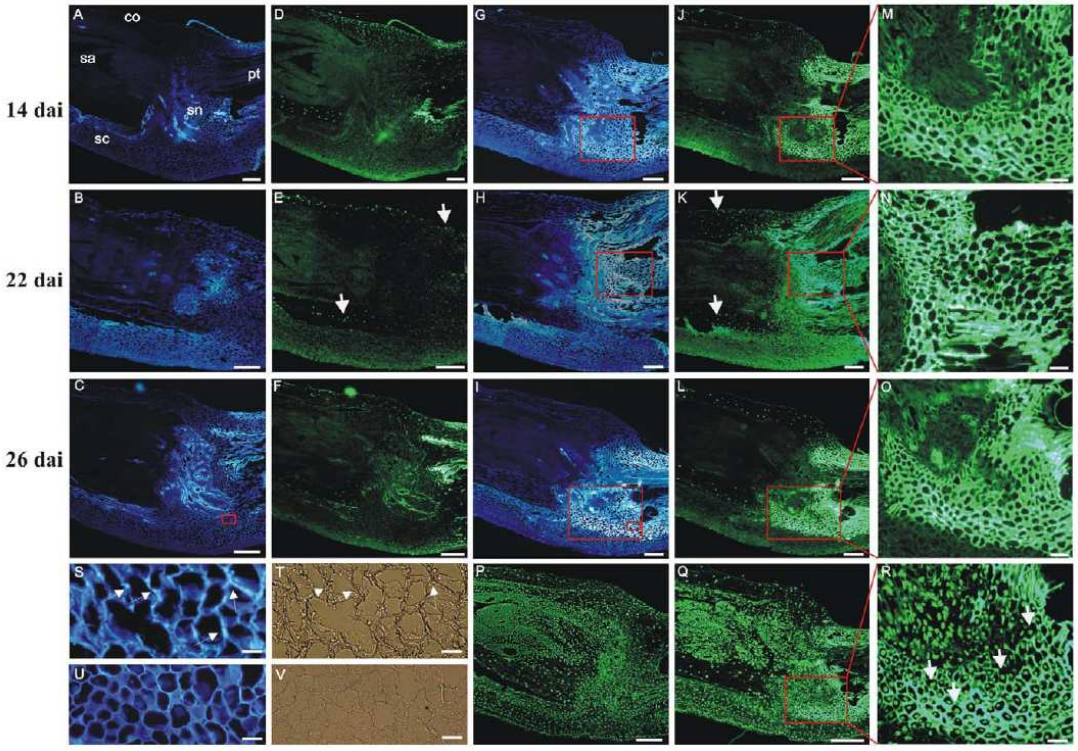

Figure 7. Histological analyses of NIL3876-*Rdg2a* barley embryos. (A) to (C) Sections of embryos grown under control conditions observed under UV excitation. (D) to (F) Sections in (A) to (C) subjected to TUNEL analysis. (G) to (I) Sections of embryos inoculated with leaf stripe isolate Dg2 and observed 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. graminea* 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 resistance 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 *Xa3* [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 = tssp, [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-Rdg2* 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 AVR_{A10}, 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* an intercellular mode of pathogenesis, are in each case recognized by membrane-anchored resistance proteins containing extracellular LRRs [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 unviable 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 latter stages of colonization by means of hydrolytic degradation of host cell walls. *Rdg2a* resistance terminates *P. graminea* 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 F_2 recombinants for the 3.47-cM *Rdg2a* marker interval ABG704-ScOPQ9, previously selected from an F_2 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]. Barley cv. Morex was used for Southern-blot experiments while the susceptible variety Golden Promise was used for transformation tests. The leaf stripe (*P. graminea*) 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 monoconidial isolates [51]. The *P. graminea* 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 658 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 95-9-3 (*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 cosmid 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 *Eco*RI and

*Kpn*I, 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-eYFP) previously used in barley transient expression studies [65]. The constructs contain the *Nbs1-Rdg2a* and *Nbs2-Rdg2a* ORFs 3′-fused with the *YFP* 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 AOBS 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 deoxynucleotidil 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′,6-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 calcofluor staining, sections were incubated in 0.01% calcofluor in PBS pH 7 for 30 min. Samples were observed with 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 calcofluor 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.
Found at: doi:10.1371/journal.pone.0012599.s003 (0.04 MB DOC)

Table S4 PCR primers used to generate constructs for barley transformation.
Found at: doi:10.1371/journal.pone.0012599.s004 (0.04 MB DOC)

Figure S1 Southern blot analysis of *Rdg2a* candidates. BamHI-digested barley genomic DNA was hybridised with probes derived from the LRR region of the NB-LRR genes.
Found at: doi:10.1371/journal.pone.0012599.s005 (8.07 MB TIF)

Figure S2 DNA sequence homologies between paralogs and alleles at the *Rdg2a* leaf stripe resistance locus. Diagrams above define the domains compared. Percent identities were determined once major insertions/deletion differences had been removed.
Found at: doi:10.1371/journal.pone.0012599.s006 (9.88 MB TIF)

Figure S3 Demonstration that the sequenced Mirco *Nbs1-rdg2a* and *Nbs2-rdg2a* genes represent alleles of the respective Thibaut genes. Markers Nbs1-14-19 and Nbs2-6-29 developed using insertion/deletion polymorphisms in the putative regulatory regions (A) co-segregated with the *Rdg2a* locus in 12 rare recombinants for the *Rdg2a* region that had been identified in the high resolution mapping population (B). Recombination points are illustrated below (C).
Found at: doi:10.1371/journal.pone.0012599.s007 (9.85 MB TIF)

Figure S4 Predicted ORF and putative protein domains encoded from the Mirco genes *Nbs1-rdg2a* and *Nbs2-rdg2a*.
Found at: doi:10.1371/journal.pone.0012599.s008 (9.84 MB TIF)

Figure S5 Alignment of the deduced LRR domain sequences of RDG2A and NB2-RDG2A. Substitution differences are boxed; those in grey and green represent conservative and non-conservative substitutions (as defined by ClustalW), respectively. The regions of the LRRs that correspond to the β-strand/β-turn motif xxLxLxx are framed and the leucine (or other aliphatic) residues that form the structural backbone of the LRR units in RDG2A are in red.
Found at: doi:10.1371/journal.pone.0012599.s009 (9.58 MB TIF)

Figure S6 DAPI staining of embryo sections analyzed for autofluorescence and by TUNEL in Figure 7. DAPI staining of nuclei was performed for embryo sections of Figure 7 A and D (A), B and E (B), C and F (C), G and J (D), H and K (E), I and L (F). Scale bars represent 200 μM.
Found at: doi:10.1371/journal.pone.0012599.s010 (9.88 MB TIF)

Methods S1 Supplementary text for Materials and Methods.
Found at: doi:10.1371/journal.pone.0012599.s011 (0.06 MB DOC)

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Author Contributions

Conceived and designed the experiments: DB CB NCC PSL GV. Performed the experiments: DB CB GC GV. Analyzed the data: DB CB GV. Wrote the paper: DB CB NCC AMS PSL GV.

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ORIGINAL PAPER

Identification and mapping of the leaf stripe resistance gene *Rdg1a* in *Hordeum spontaneum*

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Abstract Leaf stripe of barley, caused by *Pyrenophora graminea*, is an important seed-borne disease in organically grown as well as in conventionally grown Nordic and Mediterranean barley districts. Two barley segregating populations represented by 103 recombinant inbred lines (RILs) of the cross L94 (susceptible) × Vada (resistant) and 194 RILs of the cross Arta (susceptible) × *Hordeum spontaneum* 41-1 (resistant) were analysed with two highly virulent leaf stripe isolates, Dg2 and Dg5, to identify loci for *P. graminea* resistance. A major gene with its positive allele contributed by Vada and *H. spontaneum* 41-1 was detected in both populations and for both pathogen isolates on chromosome 2HL explaining 44.1 and 91.8% R^2, respectively for Dg2 and Dg5 in L94 × Vada and 97.8 and 96.1% R^2, respectively for Dg2 and Dg5 in Arta × *H. spontaneum* 41-1. Common markers in the gene region of the two populations enabled map comparison and highlighted an overlapping for the region of the resistance locus. Since the map position of the resistance locus identified in this report is the same as that for the leaf stripe resistance gene *Rdg1a*, mapped earlier in Alf and derived from the 'botanical' barley line *H. laevigatum*, we propose that leaf stripe resistance in Vada and *H. spontaneum* 41-1 is governed by the same gene, namely by *Rdg1a*, and that *Rdg1a* resistance could be traced back to *H. spontaneum*, the progenitor of cultivated barley. PCR-based molecular markers that can be used for marker-assisted selection (MAS) of *Rdg1a* were identified. An *Rdg1a* syntenic interval with the rice chromosome arm 4L was identified on the basis of rice orthologs of EST-based barley markers. Analysis of the rice genes annotated into the syntenic interval did not reveal sequences strictly belonging to the major class (nucleotide-binding site plus leucine-rich repeat) of the resistance genes. Nonetheless, four genes coding for domains that are present in the major disease-resistance genes, namely receptor-like protein kinase and ATP/GTP-binding proteins, were identified together with a homolog of the barley powdery mildew resistance gene *mlo*. Three (out of five) homologs of these genes were mapped in the *Rdg1a* region in barley and the *mlo* homolog map position was tightly associated with the LOD score peak in both populations.

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Introduction

Leaf stripe is a widespread seed-borne disease of barley caused by the fungal pathogen *Pyrenophora graminea* (Ito and Kuribayashi). The fungal mycelia survive in seeds between the parenchymatic cells of the pericarp, the hull and the seed coat, but not in the embryo. During seed germination, fungal hyphae grow intercellularly from the coleorhiza up all sides to the roots and scutellar node where they start the infection of the shoot (Haegi et al. 2008). Infection spreads into the young leaves, where it causes

longitudinal dark brown stripes between the leaf veins. Spores produced on the infected leaves of susceptible plants during flowering spread to infect nearby heads. No secondary cycles are present in this disease. The disease is particularly acute in Nordic countries (spring sowing) and in the Mediterranean's winter barley districts, where soil temperatures below 12°C during seed germination promote the infection of the rootlet. The typical symptoms, spike sterility and chlorotic stripes on leaves, which gradually extend to the full length of the leaf and finally become necrotic, lead to severe yield reductions when seed infection is high, especially in organic farming systems (Delogu et al. 1995; Mueller et al. 2003). A variation in pathogenicity among different fungal isolates on the same genetic material has been reported, and the selective pressure by the pathogen strains on the host population may explain the existence of different resistance genes (Boulif and Wilcoxson 1988; Gatti et al. 1992).

In a search for sources of resistance, 1,029 varieties and lines from various Nordic barley collections were tested for their reaction to leaf stripe (Skou and Haahr 1987). Using pedigree analysis, the resistance of about 50–100 barley cultivars was traced to a Gull × *Hordeum laevigatum* hybrid that had been crossed to introduce the *MlLa* resistance gene for powdery mildew carried by *H. laevigatum* into Gull. It was proposed that this leaf stripe resistance gene be referred to as the Vada resistance gene, because it was unconsciously introduced into many spring cultivars, together with *MlLa*, through Vada (Skou and Haahr 1987; Skou et al. 1994). The Vada resistance locus was mapped to the long arm of the chromosome 2H using a doubled haploid population derived from the cross Alf × Vogelsanger Gold (Thomsen et al. 1997) and designated *Rdg1a*. A QTL analysis for resistance against the *P. graminea* isolate Dg2 conducted on RILs derived from the cross L94 × Vada detected one major gene on chromosome 2H at the same location as *Rdg1a* (Arru et al. 2002). Based on common markers it was previously suggested that the resistance of Vada to *P. graminea* is conferred by the *Rdg1a* gene (Arru et al. 2002), which governs resistance to leaf stripe in the barley cultivar Alf (Thomsen et al. 1997). The hypothesis that Vada and Alf carry the same leaf stripe resistance gene is further supported by the observation that the two cultivars share *H. laevigatum* as an ancestor and as the donor of the powdery mildew race-specific resistance gene *MlLa*, which is in linkage with *Rdg1a* on barley chromosome 2HL (Giese et al. 1993). Vada resistance (*Rdg1a* gene) proved to be effective against two highly virulent leaf stripe isolates and against the natural field pathogen population of different barley cultivating countries, thus suggesting that *Rdg1a* may have a very wide range of effectiveness (Skou et al. 1994; Arru et al. 2003a; Mueller et al. 2003).

In addition to *Rdg1a*, other leaf stripe resistance genes have been identified. The major resistance gene *Rdg2a* located distal on the short arm of chromosome 7H (Tacconi et al. 2001) has been mapped to fine genetic resolution (Bulgarelli et al. 2004). *Rdg2a* confers complete resistance (immunity) to at least three Italian isolates of the pathogen, including the most virulent one (Dg2), but it is not effective against the isolate Dg5. The partial resistance of Proctor is conferred by a gene in the centromeric region of chromosome 7H in the spring barley cross Proctor × Nudinka (Pecchioni et al. 1996). This QTL had a major effect on the trait and has been designated as the Proctor resistance gene. Partial resistance of cv Steptoe is governed by major QTLs mapped to the long arm of chromosome 2H and on chromosome 3H (Arru et al. 2003b).

In the course of a barley germplasm screening for leaf stripe resistance sources with a wide range of effectiveness against highly virulent monoconidial isolates and the field pathogen population, *H. spontaneum* 41-1 and the barley cv Vada were among the genotypes with the highest level of resistance, while Arta and L94 were among the most susceptible (Mueller et al. 2003 and unpublished data). These genotypes were verified as being parents in two barley mapping populations (Baum et al. 2003; Marcel et al. 2007a).

In the present work QTL analysis was applied to the RILs progenies of two crosses that included *H. spontaneum* 41-1 and Vada as resistant parents, and were therefore segregating for the resistance against two highly virulent leaf stripe monoconidial isolates. In one of the crosses, L94 × Vada, a dense molecular marker map was used to precisely detect the map position of the Vada resistance. The other population, Arta × *H. spontaneum* 41-1, was used to identify resistance loci contributed by *H. spontaneum*, the wild progenitor of cultivated barley. Markers added to this latter map also enabled comparisons among the resistance loci detected in the two populations. A syntenic interval on the rice chromosome arm 4L was identified on the basis of rice orthologs of EST-based barley markers, allowing a search for possible candidate genes for *Rdg1a* resistance among the annotated rice genes. Mapping of three *Rdg1a* candidates revealed that one of them, a homolog of the powdery mildew resistance gene *mlo*, was tightly associated to the LOD peak of the resistance gene in both the populations.

Materials and methods

Plant material

Two spring barley segregating populations of RILs obtained by single-seed descent were tested with their

respective parents for leaf stripe resistance in an artificial inoculation test. One barley population (L × V) consisted of 103 F9 inbred lines obtained from a cross between L94 (leaf stripe susceptible), a two-rowed line with black and naked seeds, and Vada (leaf stripe resistant), a two-rowed cultivar with white and hulled seeds (Qi et al. 1998). The second population (A × H.sp.) comprised 194 F8 RILs of the cross between Arta (leaf stripe susceptible), a two-rowed pure line selected from the Syrian white-seeded landrace "Arabi Abiad", and *H. spontaneum* 41-1 (leaf stripe resistant), a pure line selected for its adaptation to severe drought stress conditions; this cross was originally developed to study agronomic traits associated with adaptation to Mediterranean environment (Baum et al. 2003). The cvs. Rebelle (six-rowed, winter, highly resistant), Thibaut (six-rowed, winter highly resistant to isolate Dg2 and susceptible to isolate Dg5) and Mirco (six-rowed, highly susceptible) were used as reference lines in the inoculation test. The cv. Gull was tested for resistance to leaf stripe isolates Dg2 and Dg5 to assess derivation of the Vada resistance.

Inoculation test and disease evaluation

The *P. graminea* isolates used (Dg2 and Dg5) are the most virulent in a collection of 12 Italian monoconidial isolates tested on European barley varieties (Gatti et al. 1992; Mueller et al. 2003). In the L × V population, infection scores for the isolate Dg2 were determined in a previous work (Arru et al. 2002), but in the present work 38 lines out of 103 were scored again with this leaf stripe isolate. In addition, 91 lines of L × V were screened with the isolate Dg5. In the A × H.sp. population 122 and 121 lines were, respectively tested with the isolates Dg2 and Dg5. The RILs, parents and test cvs. were inoculated using the "sandwich" method following the procedure described in Pecchioni et al. (1996).

For each line, sixty seeds were surface-sterilized in 70% ethanol for 30 s and 5% NaOCl for 10 min, rinsed thoroughly in deionized water, left to dry and then incubated in three Petri dishes (20 seeds each) between two potato dextrose agar (PDA; Liofilchem, Teramo, Italy) layers colonized by an actively growing mycelium. After 20 days of incubation in the dark at 6°C, the emerged seedlings were transplanted to pots 12 cm in diameter and grown in the greenhouse until heading at 12°C night (10 h dark) and 20°C day (14 h light at a quantum flux density of 28 $\mu E\ m^{-2}\ s^{-1}$). A randomized, complete-block design with three replications of 20 plants per line was used. At heading, infected (showing leaf stripes) and healthy plants were counted. Resistance was assessed as the incidence of infection, i.e. the percentage of infected plants.

DNA marker analysis

Genomic DNAs of the parents and each RIL was isolated by placing leaf tissues in 96 × 1.2-ml-well microtube plates. Plant material was ground using the Retsch® MM300 Mixer Mill instrument and for DNA purification the Wizard® Magnetic 96 DNA Plant System (Promega) was used following manufacturer's instructions. In order to identify molecular markers, L94, Vada, Arta and *H. spontaneum* 41-1 were screened for polymorphisms. PCRs for STS, CAPS and dCAPS analyses were performed in volumes of 20 µl, containing 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 µM of each primer, 5% DMSO, 0.5 U Taq polymerase, and 60 ng template DNA. The PCR conditions comprised one cycle of 2 min at 94°C, 36 cycles of 40 s at 94°C, 50 s at 60°C (66°C for marker TC163743), 1 min 20 s at 72°C, and a final extension 72°C for 10 min. Gel-purified PCR products were directly sequenced to confirm identity (by comparison to the original sequence), and to identify polymorphisms between the parents of the mapping populations. When amplification products were longer than 1 kb, sequencing primers were used to extend sequence reading to the whole amplified fragments. Restriction sites covering polymorphic sites were identified using the RestrictionMapper V3.0 program (http://www.restrictionmapper.org/), while primers for dCAPS analysis were identified with the program dCAPS Finder V2.0 (http://helix.wustl.edu/dcaps/dcaps.html). For CAPS and dCAPS markers, 20 µl of PCR mixture was digested overnight in a volume of 25 µl containing 1× restriction enzyme buffer, 1.5 U of restriction enzyme and 0.5 µg/µl of acetylated BSA. The resulting fragments were size fractionated in 2% agarose gels. For SSR analysis each reverse primer was 5′-tailed with the M13 forward consensus sequence. The M13-tailed reverse primers were then used in combination with the forward primers and with a standard M13 primer dye-labelled at its 5′ end (Boutin-Ganache et al. 2001). PCRs were performed in volumes of 10 µl, containing 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.2 µM of forward primer, 0.02 µM of M13-tailed reverse primers, 0.08 µM of M13 dye-labelled primer, 0.5 U Taq polymerase, and 60 ng template DNA. The PCR conditions comprised one cycle of 2 min at 94°C, 36 cycles of 45 s at 94°C, 45 s at 55°C, 1 min at 72°C, and a final extension 72°C for 7 min. Microsatellites polymorphisms were visualized using an ABI PRISM 3130xl genetic analyzer (Applied Biosystems, Foster City, CA).

The InDel-based HvCSG STS marker applied on the L × V population was obtained with primers reported in Table 1 designed from position 4,500 to position 5,205 of GenBank sequence X58339 coding for the barley chalcone synthase gene (Becker and Heun 1995). Additionally, the CAPS marker MWG2068 (Marcel et al. 2007b) and the

Table 1 STS, dCAPS (d) and CAPS markers tested for linkage to *Rdg1a*

Marker	Primer	Restriction enzyme	
		L × V	A × H.sp.
HVCSG	5′-CCTTCTCGACCGTTTATCTTCGTCATGG		
	5′-CTGCAGGGCTGCTTCAATGAGC		
NP450530(d)	5′-GCAGCGTCAGCGTGTCAAGAACCGTTCCGTCGTCA	*BseLI*	
	5′-TTCCCGGAGGACCAGACCTAC		
TC163743	5′-AAGGAGTTCAACTGGAACTTTGAA	*HinfI*	
	5′-CCAGTCATAGTCGCATACTATC		
FD526114	5′-TCTCTCATCTATGATATGATCCTAGC	*AflII*	*TasI*
	5′-CAACAGGATCAGAGAAACCATGC		

For each marker the sequence of the primers and the restriction enzyme used to detect the polymorphisms are shown

SSR markers GBM1047, GBM1462 and GBM1475 (Varshney et al. 2007) were applied on the A × H.sp. population. Sequences of barley ESTs representing putative orthologs of *Rdg1a* candidates identified on the rice chromosome 4L (see below) were also used for primer design (Table 1) using procedures previously described (Chen et al. 2009) in order to include intron sequences within the amplified genomic fragments.

Linkage analysis

For the L × V, the segregation data of 958 markers were obtained from the L94 × Vada 2006 map data of GrainGenes (http://wheat.pw.usda.gov/GG2/index.shtml) (Marcel et al. 2007b), and for A × H.sp., the segregation data of 193 markers were obtained from Baum et al. (2003). Map position of the molecular markers added in the present work was established with the software JoinMap 4.0 (van Ooijen 2006) using Kosambi's (1944) mapping function. All mapped markers were tested for the expected 1:1 segregation ratio using a χ^2 goodness-of-fit test and were joined into the corresponding linkage groups using LOD score of 3.0 or higher using the "Second Order" mapping in Join Map 4.0. Mapping of TC163743, NP450530, HVCSG and FD526114 into the chromosome 2H of the L × V population was conducted by adding the order of the microsatellite markers HVM54, GBM1200, GBM1047 GBM1462, Bmag0749 and GBM1475 as a "fixed order file" into Join Map 4.0. "Fixed order files" were not used for mapping of GBM1047, FD526114, GBM1462, MWG2068 and GBM1475 into the A × H.sp. map.

Statistical and QTL analyses

ANOVA and correlation analyses of the resistance data were performed using SYSTAT v.9 software (Systat Software Inc., CA, USA). Broad sense heritabilities ($h^2 = \delta^2 g/\delta^2 p$) were calculated for the four experiments (reaction scores to Dg2 and Dg5 in L × V and A × H.sp populations) on ANOVA results. For QTL analysis, mean data of infection scores were first used for simple interval mapping (SIM) to identify the markers most significantly associated with variation in leaf stripe resistance. To improve the QTL detection capacity, automatic co-factor selection (ACS) analysis was performed to identify markers significantly associated with leaf stripe resistance. Co-factors were then used in a multiple-QTL model (MQM) in MapQTL v. 5. The QTL analysis was repeated by selecting the markers associated with the QTLs as co-factors as described by van Ooijen (2004). Permutation tests (1,000 iterations) were performed for each experiment to determine the threshold at which the LOD score became significant ($P < 0.05$) and highly significant ($P < 0.001$) for QTL identification (van Ooijen 2004). For QTL mapping, co-segregating markers were removed from L × V.

Syntenic relationship with rice

Identification of orthologous rice genes for the markers GBM1498, GBM1462, and GBM1012 is described in Stein et al. (2007), while identification of orthologous rice genes for the marker WBE110 is described in Marcel et al. (2007a). The same procedure has been used in this work to identify a rice ortholog of the barley *Gln2* locus coding for a glutamine synthetase 2 (accession number X53580). Rice genomic sequence from the leaf stripe resistance locus syntenic region of chromosome 4 was scanned for resistance proteins of all classes, as defined in Table 2 of Hammond-Kosack and Parker (2003) by using the release 6.1 of the MSU rice genome annotation project database (http://rice.plantbiology.msu.edu/pseudomolecules/info.shtml; Ouyang et al. 2007). Initial searches were conducted using 20-kb sections and, for sections of interest, additional searches were performed using 10 kb sections. Putative barley orthologs of the rice genes were identified by Blast search in the barley gene indices at DFCI (http://compbio.dfci.harvard.edu/tgi/plant.html).

Results

Phenotypic analysis

The analysis of variance on the percentage of infected plants in the two segregating populations for the two leaf stripe isolates showed a highly significant effect of the genotype ($P < 0.001$) on the incidence of barley leaf stripe, with no significant differences observed in the replications. *H. spontaneum* 41-1 and Vada were resistant to both isolates while Arta and L94 were highly susceptible (Fig. 1). In this work, when L94 was infected with isolate Dg2 the infection score recorded (85%) (Fig. 1a) was higher than that previously observed (38%) by Arru et al. (2002). Because of this difference, resistance tests on L94 were carried out in three additional independent infection experiments though results always confirmed the higher infection score (data not shown). Figure 1 shows the distribution of the leaf stripe resistance to each isolate in the two segregating populations, calculated as percentage of infected plants. The infection score dataset for isolate Dg2 used for QTL mapping in the present work was based on 103 RILs. For 65 of these RILs the infection data for isolate Dg2 were the same as those used in the mapping experiment of Arru et al. (2002), while for the remaining 38 lines new infection experiments with isolate Dg2 were carried out in the present work. For these 38 lines, previous infection scores for isolate Dg2 (Arru et al. 2002) were contrasting with infection scores obtained for isolate Dg5 in the present work, while for the remaining 65 lines there was a substantial agreement for Dg2 and Dg5 infection values. This evidence prompted a new detailed evaluation to be carried out. As previously observed (Arru et al. 2002), the higher infection scores of the population means for Dg5 (at least in L × V) together with the higher values observed for the parents may indicate that Dg5 is a more virulent isolate than Dg2.

In the two RIL populations the distribution of resistance to both isolates deviated significantly from normality, even though fitting a U-shaped (or sigmoid shaped for L × V population infected with isolate Dg2) frequency distribution separated by a region of very low frequencies (Fig. 1). Although the phenotypic distribution could fit the segregation of a single gene (at least for distributions in Fig. 1b–d), lines belonging to intermediate resistance classes were also observed, thus raising the possibility that more than one locus could be involved in the resistance. For this reason data were subsequently processed by means of a QTL analysis.

The estimates of heritability in broad sense for resistance to isolates Dg2 and Dg5, respectively within L × V and A × H.sp populations were 0.998 and 0.997 for L × V, and 0.999 and 0.999 for A × H.sp. These high heritability values indicated that the majority of the phenotypic variance was due to genetic effects. A significant correlation was found between the disease incidence of isolate Dg2 and that of isolate Dg5 in both the L × V ($r = 0.796$; $P < 0.001$) and A × H.sp ($r = 0.941$; $P < 0.001$) populations.

Genetic mapping and QTL analysis

SSR markers GBM1047, GBM1462, GBM1475 and the CAPS marker MWG2068 were mapped on the A × H.sp. population, while the STS marker HVCSG was mapped in the L × V population (Fig. 2). These additional markers were used because, on the basis of preliminary mapping experiments, loci for resistance were shown to be localized in this distal region of chromosome 2HL. Markers cited by several authors as being linked to this region (Marcel et al. 2007b; Varshney et al. 2007) were therefore added to the two maps both to increase the marker density of the gene region and to obtain bridge markers allowing comparison of the locus position. In addition, markers derived from the barley chromosome 2H/rice chromosome 4L syntenic relationship (see below), represented by NP450530 (dCAPS marker), TC163743 and FD526114 (CAPS markers) were mapped in L × V, while FD526114 was mapped in A × H.sp. (Fig. 2; ESM Fig. S1). In this latter population, mapping of NP450530 and TC163743 was not possible because of the absence of polymorphisms between the two parents. An inverted order was observed for the markers GBM1047 and HVCSG in the two maps obtained, which was unexpected, and so segregation data for the two markers were visually scored in the two populations to control for incoherencies with the assigned map position but, even after this analysis, the results of the linkage analysis were confirmed. The different map order of the two markers could therefore depend on a genuine inversion of the two markers, or, more likely, on the lower density of markers within the A × H.sp. map not allowing for a precise map position assignment.

In the L × V population, a resistance gene with major effects for resistance to *P. graminea* isolates Dg2 and Dg5 was detected on chromosome 2HL with LOD scores of 12.9 and 42.7, respectively (Fig. 2; Table 2). The parent Vada contributed the resistance alleles for this gene to both isolates and the percentage of phenotypic variance explained was 44.1% for *Q-Vada-Dg2* and 91.8% for *Q-Vada-Dg5* (Table 2). The region of the locus conferring resistance to the two isolates was completely overlapping (the LOD score peak was included in the same marker interval) and is in agreement with the position of the one previously detected for the isolate Dg2 in Arru et al. (2002), which represents the *Rdg1a* gene. The gene conferring resistance to isolate Dg2 was in fact previously localized (Arru et al. 2002) on the chromosome 2H region

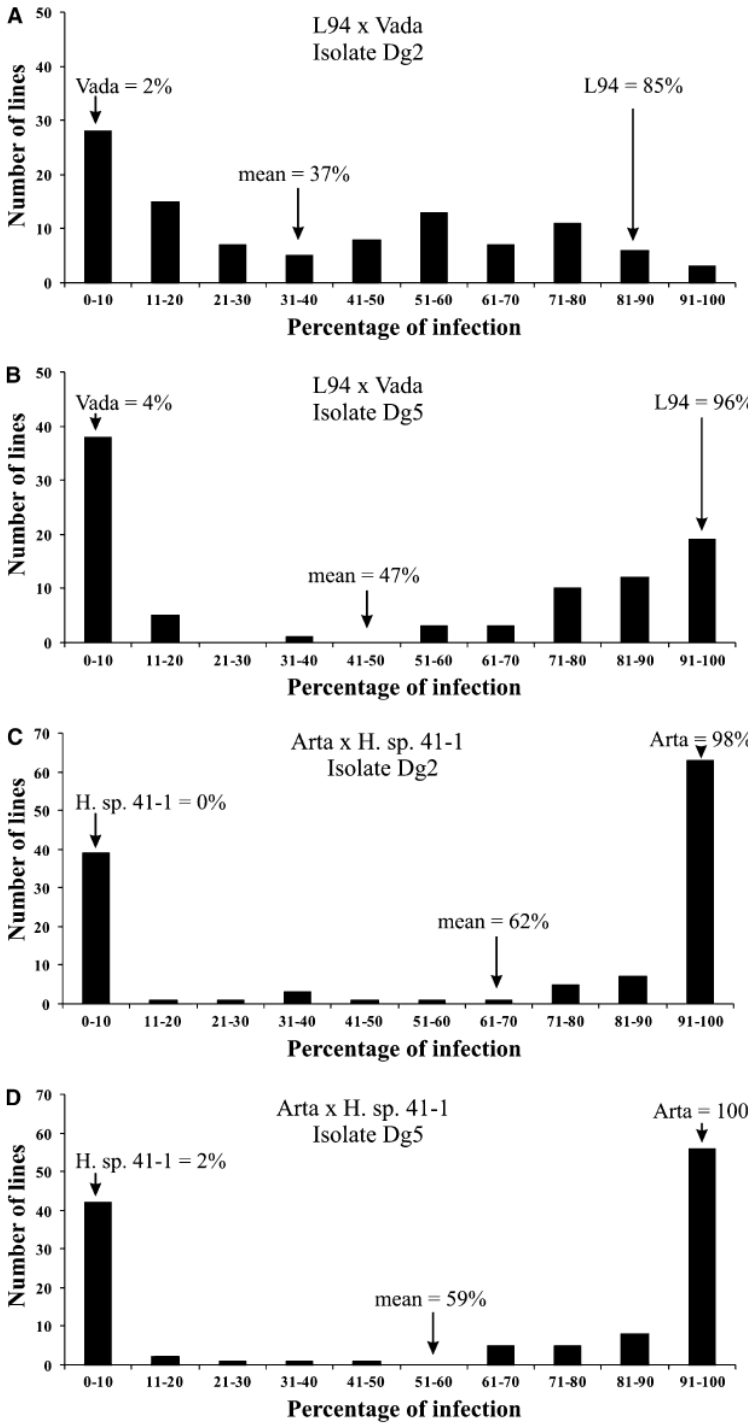

Fig. 1 Frequency distribution of phenotypic reaction to leaf stripe isolates Dg2 and Dg5 expressed as percentage of infection in RILs derived from L94 × Vada (**a**, **b**) and Arta × *H. spontaneum* 41-1 (**c**, **d**). Resistance values of the parents and means of the populations are shown and their position is indicated by arrows

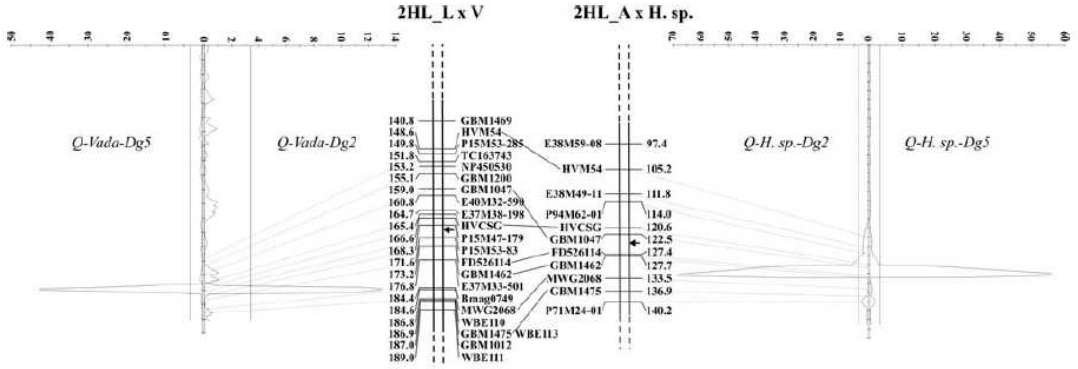

Fig. 2 Multiple-QTL model (MQM) LOD scans of barley chromosome arm 2HL where loci for leaf stripe resistance to isolates Dg2 and Dg5 were detected in the two RIL populations. *Vertical lines* indicate significance threshold for each experiment, estimated from 1,000 permutation of the data. Distances are given in Kosambi cM. Markers in common among the two populations are connected with *continuous lines*. Positions of the markers on the QTL LOD plots are indicated with *dotted lines*. *Arrows* indicate the map position of the locus for resistance to isolates Dg2 and Dg5 in A × H.sp. and for resistance to isolate Dg5 in L × V, as mapped by JoinMap 4.0

Table 2 LOD scores, percentages of phenotypic variance explained (R^2) and estimated additive effects of the detected QTLs in the RIL populations L × V and A × H.sp

Locus	Marker interval[a]	Donor	LOD	R^2 (%)[b]	Additive effect[c]
Q-Vada-Dg2	P15M53-83-FD526114	Vada	12.9	44.1	18.9
Q-Vada-Dg5	P15M53-83-FD526114	Vada	42.7	91.8	40.6
Q-H.sp.-Dg2	GBM1047-FD526114	H. sp.	68.3	97.8	48.0
Q-H.sp.-Dg2	GBM1047-FD526114	H. sp.	55.9	96.1	46.4

[a] The marker interval including the QTL peak position

[b] The amount of total trait variance explained by a QTL at this locus

[c] The positive values indicate that alleles of the gene from Vada or from *H. spontaneum* positively contributed to the resistance or reduced the severity of disease

delimited by AFLP markers E40M32-590 (this marker is presented in Fig. 2) and E42M40-644 (this marker is 1 cM distal to GBM1462). The present work therefore supports that *Q-Vada-Dg2* and *Q-Vada-Dg5* represent effects of the leaf stripe resistance gene *Rdg1a*.

In the A × H.sp. population, MQM mapping detected a resistance gene with major effects for resistance to isolates Dg2 and Dg5 on chromosome 2HL with LOD scores of 68.3 and 55.9, respectively (Fig. 2; Table 2). The resistance allele of this gene was contributed by the parent *H. spontaneum* 41-1 and the percentage of phenotypic variance explained was 97.8% for *Q-H.sp.-Dg2* and 96.1% for *Q-H.sp.-Dg5* (Table 2). Also in this mapping population, the resistance to both isolates mapped to the same location.

No additional loci were found above the threshold using MQM either in L × V or in A × H.sp. populations. The minor QTL detected by Arru et al. (2002) as a subthreshold peak (LOD = 2.04) on chromosome arm 7HL was not detected in any of the experiments in the present work.

In the two populations QTL peaks were defined by an interval of 3 cM between P15M53-83-FD526114 in L × V, and about 5 cM between GBM1047-FD526114 in A × H.sp.(Table 2; Fig. 2). The SSR marker GBM1462 is just distal to the gene region in both populations (Fig. 2). In the two populations, allelic variation at markers FD526114 and GBM1462 was associated to leaf stripe resistance, as demonstrated by the observation that alternate alleles of the two markers were the most predictive for the average level of resistance or susceptibility in the RILs from the L × V and A × H.sp. populations (Fig. 3). This locus–marker relationship was further tested by mapping the resistance gene loci as qualitative traits using JoinMap 4.0. Mapping was carried out for resistance to isolates Dg2 and Dg5 in A × H.sp. and only for resistance to Dg5 in L × V by excluding RILs with intermediate infection values (from 20 to 60%) from the mapping dataset. In A × H.sp., a locus for resistance to both isolates was mapped between markers GBM1047 and FD526114 (1.5 cM distal to GBM1047 and 2 cM proximal to FD526114), while in L × V the resistance

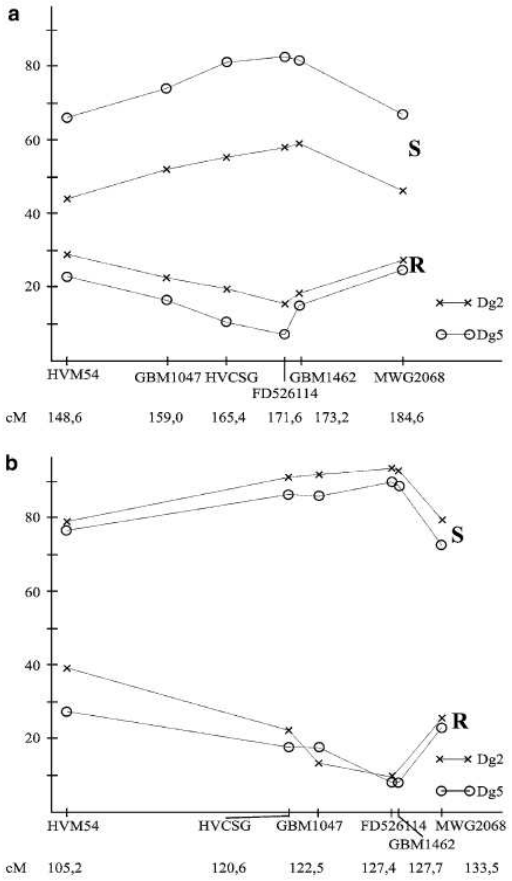

Fig. 3 Average infection response (percentage of infected plants) after inoculation with isolate Dg2 or Dg5 of lines from the L × V (**a**) and A × H.sp (**b**) populations with the resistance (*V* or *H*.sp.) or the susceptibility (L or A) alleles at molecular markers linked to *Rdg1a*. Along the *X*-axis the marker names and their distances in cM are indicated. Different scales were used to define marker distances in **a** and **b**

gene was localized between the markers P15M53-83 and FD526114 (1.0 cM distal to P15M53-83 and 2.2 cM proximal to FD526114) (Fig. 2). These JoinMap 4.0 mapping results were therefore in complete agreement with QTL mapping data.

A hypothetical origin for *Rdg1a*

Leaf stripe resistance test supports that *Rdg1a* gene in Vada can only be derived from *H. laevigatum*, because the other parent (Gull) scored as highly susceptible to the two leaf stripe isolates (Fig. 4). On the basis of the present mapping result, it can be postulated that *Rdg1a* confers resistance to the two leaf stripe isolates tested. The QTL peak for resistance against Dg2 and Dg5 was flanked by the markers HVCSG and GBM1462, common to both the populations used in the study, which corresponded to a genetic interval of 7.8 and 7.1 cM in L × V and A × H.sp., respectively (Fig. 2). This overlapping interval therefore, strongly supports the hypothesis that leaf stripe resistance in Vada and *H. spontaneum* 41-1 is governed by the same gene, namely, by *Rdg1a*, and that *Rdg1a* resistance derived from *H. laevigatum* could be traced back to *H. spontaneum*, the progenitor of cultivated barley (Fig. 4). This hypothesis is further supported by the nearly identical level of resistance of the two barley genotypes (*H spontaneum* 41-1 and Vada) to the two leaf stripe isolates (Figs. 1, 4).

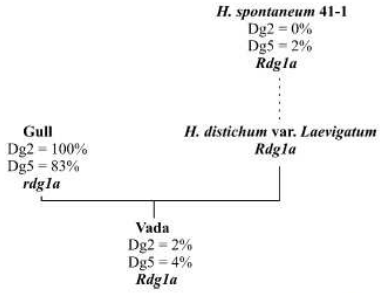

Fig. 4 Diagram depicting the proposed derivation of *Rdg1a* from *H. spontaneum* 41-1

Syntenic relationship with rice

A syntenic relationship between the long arm of the chromosome 2 region bearing the leaf stripe resistance locus identified in this work and the rice chromosome arm 4L was highlighted in previous studies (Marcel et al. 2007a; Stein et al. 2007; Chen et al. 2009). Figure 5 depicts the syntenic relationship between this barley chromosome region and the corresponding region of rice chromosome arm 4L. Rice orthologs of the EST-based SSR markers GBM1498, GBM1462 and GBM1012 were identified by using the MoMaVis program (http://pgrc.ipk-gatersleben.de/transcript_map/momavis.php; Stein et al. 2007), while information on the rice homolog of the barley EST marker WBE110 was taken from Table S1 of Marcel et al. (2007a). For the barley gene *Gln2*, a rice ortholog with a high level of similarity ($E = 2.10^{-213}$) was identified in the TIGR (The Institute for Genomic Research Rice Genome Annotation project, http://rice.tigr.org, release 6.1) locus Os04g47066. Markers Gln2 and GBM1498 were not directly mapped in the L × V population but their map position was extrapolated on the basis of loci shared between two maps: the map position of Gln2 was assigned by comparing L × V with the consensus map of Marcel et al. (2007b), while GBM1498 was assigned by comparing

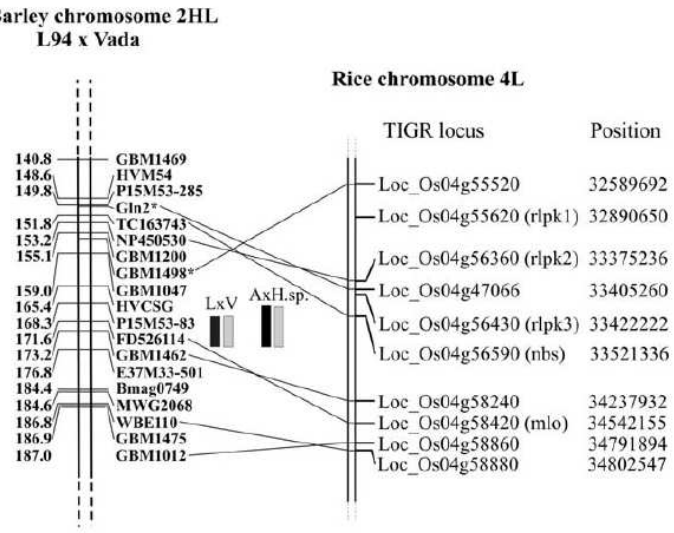

Fig. 5 Alignment of the L94 × Vada genetic linkage map covering the *Rdg1a* region on barley chromosome 2HL with the homologous region on rice chromosome 4L. *Asterisks* denoted markers whose map position was extrapolated on the basis of loci shared between two maps. Distances are given in Kosambi cM. *Black and grey rectangles* indicate the significant LOD interval for resistance, respectively to isolate Dg2 and Dg5 in the two segregating populations. The eight rice orthologs identified are connected with lines to the corresponding barley homologs and define a rice physical contig of 2.219409 Mb. The column position indicate, in bp, the 5′ end of the corresponding rice locus on the Nipponbare chromosome arm 4L. *rlpk1, rlpk2, rlpk3, nbs* and *mlo* are codes for *Rdg1a* candidates in rice, as indicated in Table 3

L × V with the consensus map of Varshney et al. (2007), as hereafter described. Marker Gln2 was mapped 0.022 cM distal to the AFLP marker P15M53-285 in a barley consensus map (Marcel et al. 2007b) and GBM1498 was mapped between markers GBM1200 and GBM1047 (0.9 cM from the first and 4.0 cM from the second marker, respectively) in a microsatellite consensus map of barley (Varshney et al. 2007). We attempted to map these two markers in the two segregating populations used in the study but, for Gln2, no SNP or In/Del polymorphisms were identified after amplification and sequencing of about 3,160 bp of genomic sequences amplified from the four parents using primers designed on the accession number X53580 (data not shown). Similarly, amplification products of the SSR marker GBM1498 were monomorphic between the parents of the two segregating populations.

The five barley EST-based markers for which a rice ortholog was identified, allowed alignment of the L × V map to a rice physical contig of about 2.212 Mb that includes the *Rdg1a* syntenic region of barley (Fig. 5). Inspection of all predicted genes annotated in the leaf stripe resistance syntenic interval in rice revealed the presence of 507 sequences coding for putative expressed proteins, 32 putative retrotransposons and 9 putative transposons. Among the 507 putative proteins genes, four were identified as coding for domains conserved within the major classes of disease-resistance proteins, three genes coding for receptor-like protein kinases and one gene coding for an ATP/GTP-binding (or nucleotide-binding sites, NBSs) protein. In addition, also an *Mlo*-like protein was present in this region (Table 3). Putative barley orthologs of these sequences were identified by searching the barley gene indices (Table 3), thus enabling development of dCAPS/CAPS markers for three of them (TC163743, NP450530 and FD526114) (ESM Fig. S1) and assignment of their map position within the *Rdg1a* genomic region (Fig. 5). For two barley ESTs, TC191004 and BY841818, it was not possible to obtain the corresponding markers. In TC191004, no SNPs were detected even after sequencing approximately 1,300 bp in the four parents, and in BY841818, the presence of multiple amplification fragments, despite the use of five different primer combinations, did not allow sequencing of the amplification products. Among the candidates tested FD526114, a sequence coding for an *Mlo*-like protein, was the most tightly associated with the leaf stripe resistance locus (ESM Fig. S1; Fig. 5) and its map position was under the QTLs LOD plots in both the populations, but not coincident with the map position of the QTL peak.

Discussion

In the present study, two barley populations segregating for leaf stripe resistance were evaluated for their response to

Table 3 Rice genes encoding domains conserved in disease-resistance proteins identified in the *Rdg1a* rice syntenic region

Code[a]	Putative function	Position[b]	Rice locus	Barley homolog[c]	*E* value[d]
rlpk1	Receptor-like protein kinase	32,890,650	Loc_Os04g55620	TC191004	9.6E-86
rlpk2	Receptor-like protein kinase	33,375,236	Loc_Os04g56360	NP450530	9.6E-124
rlpk3	Receptor-like protein kinase	33,422,222	Loc_Os04g56430	BY841818	1.2E-87
nbs	ATP/GTP-binding protein	33,521,336	Loc_Os04g56590	TC163743	2.8E-143
mlo	*Mlo*-like protein	34,542,155	Loc_Os04g58420	FD526114	1.4E-42

[a] Identification code reported in Fig. 5

[b] Position in bp for the 5′ end of the corresponding rice locus on the Nipponbare chromosome arm 4L

[c] Best barley homologs retrieved after Blast search of barley gene indices with the rice EST sequences

[d] *E* value obtained with the Blast alignment of rice sequences against the barley gene indices

two highly virulent *P. graminea* isolates in order to identify resistance loci. In the two populations only one genomic region, defined by a marker interval that included the LOD score peaks, was identified as responsible for leaf stripe resistance. This result is also supported by the high portion of phenotypic variance explained by the loci identified and from a previous study on the Vada resistance to leaf stripe isolate Dg2 in which only one major QTL was detected on chromosome 2H (Arru et al. 2002). The genomic region of resistance loci towards the two isolates was coincident in L × V and A × H.sp. populations; in addition, no recombinant lines for resistance to Dg2 and Dg5 (i.e. highly resistant to one isolate and highly susceptible to the other) were identified in the RILs analysed in either populations, thus supporting that the same gene is responsible for resistance to both isolates.

In this work we demonstrated that *H. spontaneum* accession (*H. spontaeum* 41-1) possesses the leaf stripe resistance gene *Rdg1a*. *H. spontaneum* 41-1 is a line selected from an accession originally collected in Israel, Beit Shean Valley, at an altitude ranging between 225 and 150 m below sea level (S. Grando, personal communication). The presence of the gene in this accession suggests that *Rdg1a* is present with some degree of frequency (currently unknown) within the *H. spontaneum* gene pool, and that, as a consequence, *H. spontaneum* accessions may have contributed the *Rdg1a* resistance allele to *H. distichum laevigatum*. It is known that *H. spontaneum* is a rich source of genes that impart resistance against important barley diseases and a high frequency of resistance (60–98%) to leaf blotch, leaf rust, net blotch and powdery mildew has been found in accessions from Jordan and Israel (Fetch et al. 2003). Although no studies have been performed to evaluate the frequency of leaf stripe resistance genes in *H. spontaneum*, this disease is typical of Mediterranean environments and leaf stripe is definitely present in the fertile crescent where *H. spontaneum* occurs (Yahyaoui 2004; Tunali 1995; Golzar 1995). Since the presence of the pathogen should increase selection for disease resistance, it is likely that an overlapping of the *P. graminea* and *H. spontaneum* areas may have lead to an increased frequency of leaf stripe resistance genes in the *H. spontaneum* gene pool. Very little information is available about the ancestry and provenance of *H. laevigatum* (Skou and Haahr 1987) or about the possibility of having introgressed genes from *H. spontaneum*. However, the fact that *H. laevigatum* is a landrace of *H. vulgare* ssp. *vulgare* suggests that no crossing barriers would have limited gene transfer between *H. spontaneum* and *H. laevigatum* (Asfaw and von Bothmer 1990). It is therefore possible that *Rdg1a* in *H. laevigatum* is derived from *H. spontaneum*.

A QTL for partial resistance to leaf stripe isolates Dg2 and Dg5 derived from the barley cultivar Steptoe was mapped to the long arm of chromosome 2H (Arru et al. 2003b); the peak marker of this QTL was the molecular marker Pcr1 that, on the basis of the barley consensus map of Marcel et al. (2007b), is 4.3 cM distal to GBM1462. This marker relationship therefore excludes that the resistance of Vada and the Steptoe QTL is conferred by alleles of the same *Rdg1a* gene but support the hypothesis that this region of barley chromosome 2H is enriched of sequences conferring resistance to the leaf stripe pathogen *P. graminea*.

In a previous work, only AFLP markers were identified as associated with the *Rdg1a* gene (Arru et al. 2002). In this work, the PCR-based markers HVCSG, FD525114 and GBM1462 demonstrated to efficiently predict the resistant/susceptible phenotype within the RILs analysed. These markers can therefore be used for marker-assisted selection (MAS) of *Rdg1a* in segregating populations when using Vada or *H. spontaneum* 41-1 as the donor of *Rdg1a* leaf stripe resistance. The same is true when using some of the many two-rowed spring varieties that were indicated as possessing the Vada resistance gene (Skou et al. 1994; Kraakman et al. 2006). In addition to their use in MAS, these markers should enable recombinant screening in large F2 segregating populations with the aim of mapping the *Rdg1a* gene at a higher genetic resolution. The region of the

long arm of chromosome 2 belongs to the 2L1.0 section defined as a gene-rich and highly recombinogenic region (Dilbirligi et al. 2005) with physical to genetic distance estimates of 1.1 Mb/cM (Künzel et al. 2000). This should facilitate the fine-mapping procedure through the identification of markers with a tight physical association with the *Rdg1a* gene.

Previous studies highlighted a syntenic relationship between the *Triticeae* chromosome 2 and the rice chromosomes 4 and 7 (Moore et al. 1995; Devos 2005). This syntenic relationship was in agreement with the present study for eight barley EST marker loci in the *Rdg1a* genomic region for which the corresponding rice homologs were identified. Except for two possible discontinuities in the barley–rice colinearity that can be explained by an inversion of segments, the marker order in the barley map was in agreement with the order of the predicted genes on rice chromosome 4. Two possible inversions were observed in the order of the EST-based markers Gln2-GBM1498 and WBE110-GBM1012 (Fig. 5). Since the map position of the first two markers (Gln2 and GBM1498) was extrapolated and not assigned by segregation mapping, the first inversion observed may be due to inaccuracies of their marker-locus assignment. Nonetheless, inversion events in the region that includes the marker HVM54 were previously observed in a barley–rice colinearity analysis for this region of chromosome 2HL (Chen et al. 2009), supporting that the one observed in this work represents a genuine rearrangement. The second possible inversion refers to tightly associated loci (distance between WBE110 and GBM1012 is 0.2 cM) and can therefore represent a small translocation/inversion, like those observed when saturating regions of barley resistance genes *Rph7* and *Rph5* (Brunner et al. 2003; Mammadov et al. 2005). Until now, using the rice genome sequence for the identification of *Triticeae* resistance genes during map-based cloning has been a rather fruitless approach. A homologue of the barley *Rpg1* kinase-encoding stem rust resistance gene, for example, is absent from the rice syntenic region and the whole rice genome (Han et al. 1999; Brueggeman et al. 2002) and similarly, no candidates for the barley leaf stripe resistance gene *Rdg2a*, which maps to chromosome arm 7HS, were identified in the syntenic region of rice chromosome 6 (Bulgarelli et al. 2004). Nonetheless, with the double purpose of identifying additional markers derived from rice for the *Rdg1a* region, together with possible candidates, a search for genes containing domains conserved within the major classes of disease-resistance proteins was conducted for the rice *Rdg1a* syntenic region. Analysis of the Nipponbare rice genes annotated into the 2.219 Mb leaf stripe resistance syntenic interval in rice did not reveal sequences strictly belonging to the major class (nucleotide-binding site plus leucine-rich repeat) of resistance genes. A homolog of the barley powdery mildew resistance gene *mlo*, which maps to barley chromosome 4H (Hinze et al. 1991), was identified within the rice *Rdg1a* syntenic region. This was the only candidate to map under the QTLs LOD plot area. High-resolution genetic mapping of the locus will clarify whether this gene represents an *Rdg1a* candidate, or its map localization is a positional coincidence. Furthermore, other rice EST derived from the interval defined by Loc_Os04g56590 and Loc_Os4g58240 may enable the generation of additional genetic markers in barley that are closer to *Rdg1a*.

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