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ORIGINAL PAPER

Haplotype variability and identification of new functional alleles at the *Rdg2a* leaf stripe resistance gene locus

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Abstract The barley *Rdg2a* locus confers resistance to the leaf stripe pathogen *Pyrenophora graminea* and, in the barley genotype Thibaut, it is composed of a gene family with three highly similar paralogs. Only one member of the gene family (called as *Rdg2a*) encoding for a CC-NB-LRR protein is able to confer resistance to the leaf stripe isolate *Dg2*. To study the genome evolution and diversity at the *Rdg2a* locus, sequences spanning the *Rdg2a* gene were compared in two barley cultivars, Thibaut and Morex, respectively, resistant and susceptible to leaf stripe. An overall high level of sequence conservation interrupted by several rearrangements that included three main deletions was observed in the Morex contig. The main deletion of 13,692 bp was most likely derived from unequal crossing over between *Rdg2a* paralogs leading to the generation of a chimeric Morex *rdg2a* gene which was not associated to detectable level of resistance toward leaf stripe. PCR-based analyses of genic and intergenic regions at the *Rdg2a* locus in 29 *H. vulgare* lines and one *H. vulgare* ssp. *spontaneum* accession indicated large haplotype variability in the cultivated barley gene pool suggesting rapid and recent divergence at this locus. Barley genotypes showing the same haplotype as Thibaut at the *Rdg2a* locus were selected for a *Rdg2a* allele mining through allele re-sequencing and two lines with polymorphic nucleotides leading to amino acid changes in the CC-NB and LRR encoding domains, respectively, were identified. Analysis of nucleotide diversity of the *Rdg2a* alleles revealed that the polymorphic sites were subjected to positive selection. Moreover, strong positively selected sites were located in the LRR encoding domain suggesting that both positive selection and divergence at homologous loci are possibly representing the molecular mechanism for the generation of high diversity at the *Rdg2a* locus in the barley gene pool.

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Introduction

Plant resistance to microbial pathogens is mediated by Resistance (*R*) genes whose protein products allow intracellular recognition of effector proteins. The recognition events are mostly mediated by a class of receptor proteins that commonly contain nucleotide-binding (NB) and leucine-rich repeats (LRR) domains (Dodds and Rathjen

2010). The NB domain seems to act as a molecular switch which, after the interaction between the R protein and its pathogen effector(s), activates a signal transduction leading to the induction of plant defense reactions (Lukasik and Takken 2009; Rairdan et al. 2008; Tameling et al. 2006; Ueda et al. 2006; Howles et al. 2005). The LRR domain has been identified as being involved in the recognition of pathogen effectors (Rafiqi et al. 2009; Caplan et al. 2008; Liu et al. 2007; Dodds et al. 2001; Jia et al. 2000). At the amino-terminal region, a consistent group of resistance proteins also carry a toll-interleukin receptor type (TIR) or a coiled-coil (CC) domain which plays an important role in oligomerization processes (Glowacki et al. 2011; Shen and Schulze-Lefert 2007).

R genes are frequently not effective over long time periods because of genetic adaptation of pathogens that can 'overcome' a specific *R* gene by avoiding R protein-mediated recognition. Therefore, plant resistance genes are subjected to selection pressure for diversification to generate new functional variants. Typically, diversifying selection was observed in the LRR domain, particularly in the xxLxLxx motif of individual LRR units, proposed to form a solvent-exposed β-sheet on the concave surface. It was suggested that, in cases of a direct interaction of the *R* gene product with the pathogen effector, these amino acids confer biochemical specificity to the interaction (Bella et al. 2008; Dodds et al. 2001; Jones and Jones 1997). Diversifying selection in the LRR domain has been reported for intracellular CC/TIR-NB-LRR R proteins encoded by allelic variants of *Arabidopsis RPP13*, flax *L*, wheat *Pm3* and barley *Mla* genes because non-synonymous substitutions are overrepresented compared with changes in synonymous sites (Ellis et al. 1999; Rose et al. 2004; Yahaiaoui et al. 2006; Seeholzer et al. 2010). In contrast, the N-terminal domain is usually highly conserved in allelic series of *R* genes. Examples are represented by the MLA or PM3 powdery mildew resistance proteins (Bieri et al. 2004; Yahaiaoui et al. 2006).

NB-LRR genes frequently occur in clusters and this peculiar genomic organization can facilitate allelic evolution (Qu et al. 2006; Yahiaoui et al. 2004; Jorgensen 1994). The evolution of new alleles is driven primarily by point mutations, which are then reshuffled by unequal crossing over and illegitimate recombination or gene conversion, either within or between genes (Michelmore and Meyers 1998; Leister 2004; Wicker et al. 2007). These processes can therefore create new functional alleles that, once uncovered, can be made available for resistance breeding processes.

Comparison of DNA sequence of resistance loci in different varieties within a species can provide insight into the evolution of *R* loci and uncover new allelic variants. This is the case of the barley leaf rust resistance locus *Rph7* that significantly differs in the size and composition of the intergenic regions and in the number of genes between resistant and susceptible cvs., supporting a rapid and recent divergence in this region of the barley genome (Scherrer et al. 2005). Genetic variation caused by allelic diversity at *R* gene loci was recently investigated for different *R* genes also using allele mining strategies. Recent allele mining approaches in potato, wheat and barley have demonstrated efficient identification of new resistance specificities allowing an expansion of the repertoire of functional *R* genes (Wang et al. 2008; Bhullar et al. 2009, 2010; Seeholzer et al. 2010; Kumar et al. 2010). Studies of such type also demonstrated that positive selection, particularly at the level of the LRR domain (Bhullar et al. 2010; Seeholzer et al. 2010; Zhou et al. 2007) but also in the N-terminal domains (Loutre et al. 2009), plays an important role in generating new resistance specificities.

Recently the *Rdg2a* gene, conferring resistance to the seed-borne barley leaf stripe pathogen *Pyrenophora graminea* isolate *Dg2*, has been cloned (Bulgarelli et al. 2010). This *R* gene resides in a locus containing three highly similar paralogs (*Rdg2a*, *Nbs2-Rdg2a* and *Nbs3-Rdg2a*) encoding for CC-NB-LRR proteins. The high level of sequence similarity among the three members indicated relative recent gene duplication as the driving force of the generation of the *Rdg2a* locus and the sequence comparison of the three members showed that they are subjected to diversifying selection.

In addition to *Rdg2a*, other leaf stripe resistance genes/QTLs have been identified. The *Rdg1a* resistance gene was identified in the *H. vulgare* cv. Vada and in the *H. vulgare* ssp. *spontaneum* accession 41-1. The partial resistance of Proctor is conferred by a QTL localized 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 identified in the cv. Steptoe is governed by major QTLs mapped to the long arm of chromosome 2H and on chromosome 3H (Arru et al. 2003b).

Even considering all the above-mentioned leaf stripe resistance loci, to date, *Rdg2a* (Bulgarelli et al. 2004, 2010) and *Rdg1a* (Biselli et al. 2010) represent the only genetically mapped major resistance genes and the only sources of leaf stripe resistance used in breeding programmes. Thus, given the restricted number of leaf stripe resistance genes currently available, the discovery of new resistance sources is needed to limit the spread of the pathogen.

In this work the sequences of the *Rdg2a* locus were compared in the resistant cv. Thibaut and in the susceptible cv. Morex and intraspecific rearrangements including large deletions were identified. Subsequent PCR-based haplotype analyses in different leaf stripe resistant and susceptible

barley genotypes revealed extensive haplotype variation together with the conservation of the Thibaut haplotype in barley varieties with different origin. Allele mining of the *Rdg2a* gene in barley genotypes carrying the Thibaut haplotype indicated an overall high level of sequence identity despite the presence of sites subjected to positive selection.

Materials and methods

Plant materials, *Pyrenophora graminea* isolates and infection

Since an established standard differential cultivar set for differentiating *P. graminea* isolates does not exist, eight leaf stripe isolates representing the most virulent ones of a collection of twelve isolates were used: *Dg1*, *Dg4*, *Dg9*, *Dg10*, *Dg12*, *Dg19* and, among them, the most virulent *Dg2* and *Dg5* (Gatti et al. 1992). Among the eight leaf stripe isolates considered, *Rdg2a* confers immunity (complete resistance) to five isolates (*Dg1*, *Dg9*, *Dg10*, *Dg12* and *Dg19*) in addition to isolate *Dg2*, while it is not effective toward isolates *Dg5* and *Dg4*. Therefore, the eight isolates used in the study were suitable to test the functionality of newly identified *Rdg2a* alleles both in terms of increased or decreased effectiveness. Seeds were surfaced-sterilized in 70 % ethanol and 5 % sodium hypochlorite and inoculated with the isolates using the "sandwich" technique (Pecchioni et al. 1996). For each barley genotype/leaf stripe isolate interaction, three biological replicates of 20 plants each were analyzed. The Fisher's least significant difference (LSD) method was applied for comparing treatment group means after the ANOVA using SYSTAT 12 software.

PCR and sequencing

Sequencing of the BAC clones HVVMRXALLhA0425O23 (AC248662.1) and HVVMRXALLhA0054BO4 was performed as previously indicated (Steuernagel et al. 2009; IBSC 2012). The PCR-based molecular markers screening was performed on 40 ng of genomic DNA of the different barley genotypes and Morex BAC146G20. PCR amplifications were carried out with GoTaq® DNA Polymerase (Promega) in 20 µl PCR mix supplemented with 5 % dimethyl sulfoxide. The amplification conditions were 94 °C per 2 min followed by 37 cycles of 94 °C per 40 s, 60 °C per 50 s and 72 °C per 1 min per kb and then a final extension of 72 °C per 10 min. Primers used to amplify genomic regions at Thibaut and Morex *Rdg2a* locus are reported in Table S1. For the sequencing of *Rdg2a* alleles, three overlapping regions of about 1,500 bp were amplified from cvs. Rebelle, Galleon, Acuario, Haruna Nijo and Optic genomic DNA using specific primers designed on Thibaut *Rdg2* (Table S2). Amplicons underwent gel electrophoresis and, after purification by the Wizard® SV Gel and PCR Clean-Up System (Promega), were directly sequenced. Sequencing reactions were performed by using ABI BigDye Terminator version 3.1 (Applied Biosystem) in forward and reverse directions with 2.5 ng of DNA each 100 bp. Amplicon sequencing was accomplished with PCR primers used for amplification and internal primers (Table S2).

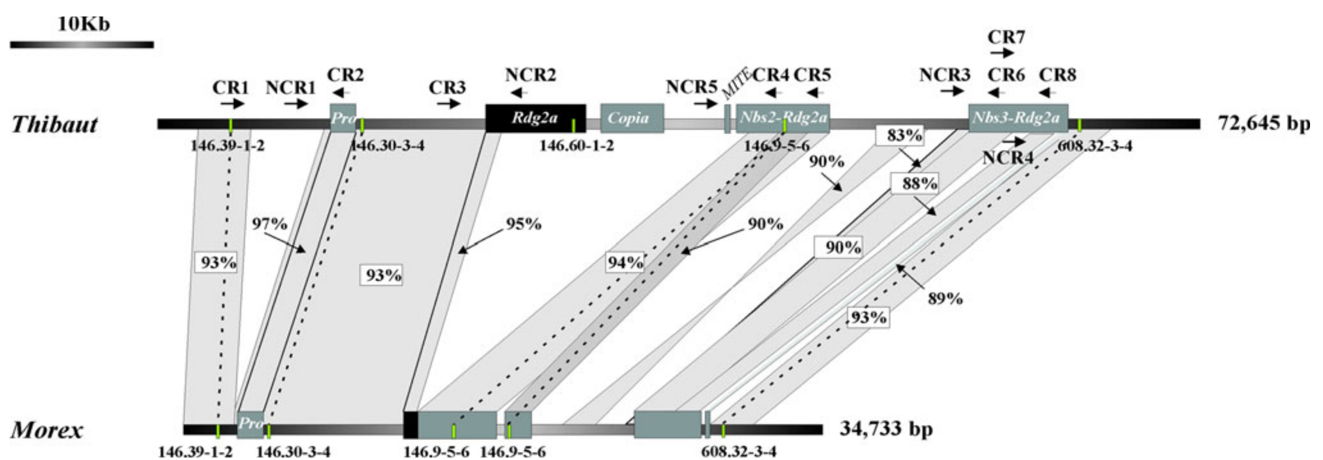


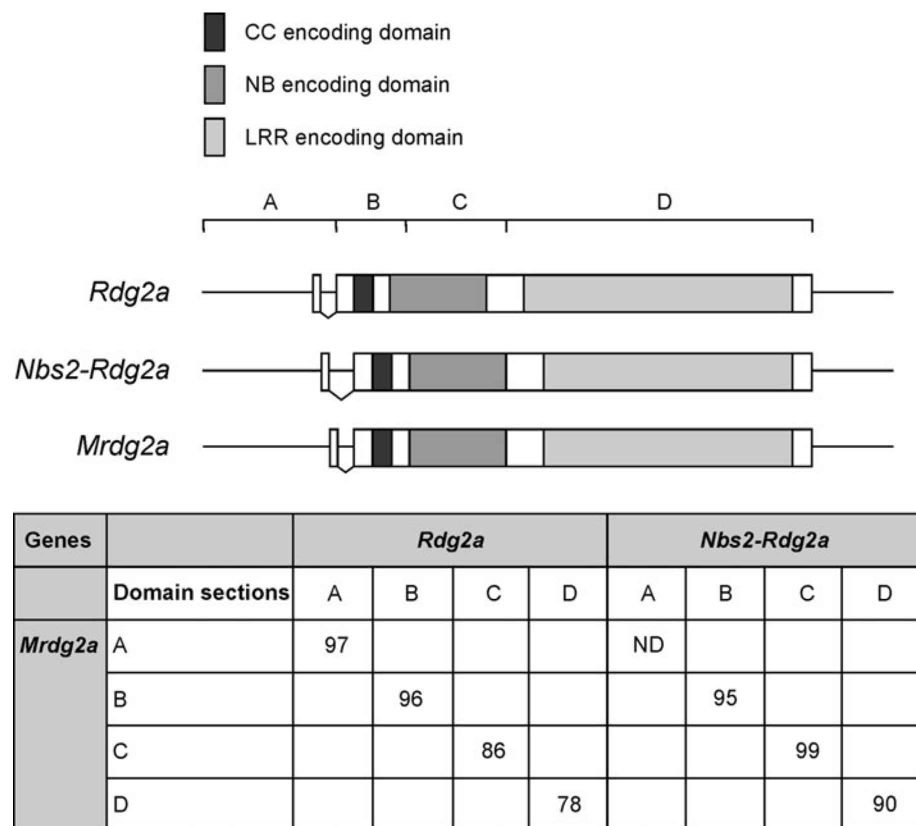
Fig. 1 Comparison between the Morex 34,733-bp long and the Thibaut 72,645-bp long contigs. Genes are indicated as in Bulgarelli et al. (2010); Pro and Copia indicate a pepsin-like aspartic protease gene and a LTR retrotransposon belonging to Copia/Ty1 family, respectively. The regions sharing over 80 % identity in nucleotide sequence among Morex and Thibaut syntenic regions were *shadowed, with the numbers* indicating the sequence identity level. Blast2seq algorithm was used for the comparison. *Arrows above the Thibaut contig* represent the positions of the primers utilized for the PCR-based haplotypic analysis. The positions of five markers present in the Thibaut contig are indicated with *vertical green bars* and the name of the markers; syntenic position of the markers in the Morex contig are connected with *dotted lines*. *White triangles* indicate the three main deletions (color figure online)

Expression analyses

RNA for expression analyses was extracted using TRIZOL® Reagent (Life Technologies), treated by DNaseI (DNA-*free*™ Kit, Ambion) and analyzed with the 2100 Bioanalyzer (Agilent). Assessment of Morex *rdg2a* (*Mrdg2a*) expression level was obtained in a two-step RT-PCR using total RNA from infected and control leaves and from embryos at 7, 14 and 22 dai (days after infection) with *P. graminea* isolate *Dg2* and after growth on sterile moist filter paper (control) at the same time-points. Two-step quantitative RT-PCR for *Mrdg2a* was performed on total RNA of control and infected embryos at 7, 14 and 22 dai. Sybr GreenER qPCR SuperMix for ABI PRISM (Invitrogen) was used and two biological replicates with three technical replicates for each time point and treatment were analyzed. Reactions were performed in the real time PCR thermal cycler 7300 Real Time PCR System (Applied Biosystem) and *Mrdg2a* expression levels in control and inoculated embryos at the same time-points of growth were compared by the use of the SDS 7300 absolute quantification software (Applied Biosystem). The expression analysis of Acuario, Galleon, Optic, Haruna Nijo, Rebelle, *H. vulgare* ssp. *spontaneum* 41-1, Nudinka, Passport and Federal alleles was carried out with total RNA extracted from young leaves. The initial RT reaction was carried out with 400 ng of RNA and the Superscript II reverse transcriptase kit (Invitrogen). Barley β *Actin* (AY145451.1) was used for RT-PCR and qRT-PCR normalization. PCR conditions were as indicated above for genomic DNA, with the exception of the number of cycles: 30 cycles for the *Rdg2a* alleles and 24 for *Actin*. Primers used for gene expression analyses are listed in Table S3.

Computational analyses

Sequence assembly was performed using ContigExpress tool of Vector NTI software package (Invitrogen). Sequence comparisons were carried out with blast2seq algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=megaBlast&BLAST_SPEC=blast2seq) and ClustalX software (Thompson et al. 1997) was used for sequence alignments which were further analyzed and edited with Genedoc program (http://www.nrb-sc.org/gfx/genedoc/index.html). The different R protein domains (CC, NB and LRR) were determined as previously described (Bulgarelli et al. 2010). The coding sequences of the *Rdg2a* alleles were used in phylogenetic analyses by PAML4 program (Yang 2007) for the estimation of the non-synonymous/synonymous ratio (k_a/k_s). The LRT based on the beta null model M7, comparing M7 and M8 was employed. Neighbor joining (NJ) phylogenetic trees for PAML analysis were calculated using MEGA4 (Tamura et al. 2007).



Genes		*Rdg2a*				*Nbs2-Rdg2a*			
	Domain sections	A	B	C	D	A	B	C	D
Mrdg2a	A	97				ND			
	B		96				95		
	C			86				99	
	D				78				90

Fig. 2 DNA sequence similarities between Thibaut *Rdg2a, Nbs2-Rdg2a* and Morex *rdg2a* (*Mrdg2a*) in different regions of the coding and non-coding sequences. *Diagrams above the table* define the domains compared. Percentage of sequence identity obtained by blast2seq algorithm is indicated (*ND* no detectable level of sequence similarity)

Results

Comparison of the *Rdg2a* locus in Thibaut and Morex reveals genome rearrangements

A collection of contigs assembled from sequences of barcoded pooled BACs from the *Dg2*-susceptible cv. Morex (Steuernagel et al. 2009) were searched for the 146G20 BAC clone, previously identified as spanning the *Rdg2a* locus found in the leaf stripe isolate *Dg2* resistant cv. Thibaut (Bulgarelli et al. 2010). The 146G20 clone was not sequenced, but within an available assembly (http://phymap.ucdavis.edu:8080/barley/; Barley PhyMap version August 2007) the clone was placed in the barley contig 9780 where two BAC clones (HVVMRXALLhA0425O23 and HVVMRXALLhA0054BO4) overlapping 146G20 were sequenced. Eleven contig sequences from 425O23 and one from 0054BO4 were assembled in a 34,733-bp long contig which was aligned, using blast2seq algorithm (http://blast.ncbi.nlm.nih.gov), to the 72,645-bp long Thibaut contig carrying the *Rdg2a* locus (Gene Bank accession number HM124452; Bulgarelli et al. 2010). The alignment revealed an overall high level of sequence conservation interrupted by several rearrangements (Fig. 1). Morex contig contained, in a collinear order, five markers present in Thibaut contig (from 146.39-1-2 to 608.32-3-4 in Fig. 1). Nonetheless, these markers spanned 52,034 bp in Thibaut contig while in Morex the distance between the two distal markers was 29,551 bp, underlining the presence of large deletions in the susceptible genotype. The size difference in the two genotypes is in agreement with previous Southern blot experiments conducted on *Bam*HI-digested genomic DNA hybridized with an *Rdg2a*-specific probe in which the size of Morex hybridization bands was more than 30 kbp smaller with respect to Thibaut (supplementary Fig. 1 in Bulgarelli et al. 2010). Three main deletions of 4,829, 13,692 bp and 2,705 were observed in Morex sequence (Fig. 1). The largest deletion (13,692 bp) expanded from base 555 downstream of the start codon of the *Rdg2a* gene (position 23,629 in HM124452), to base 562 downstream the start codon of the *Nbs2-Rdg2a* gene (position 37,321 in HM124452). This deletion most likely led to the generation of a chimeric gene between *Rdg2a* and *Nbs2-Rdg2a* establishing the Morex *rdg2a* gene (*Mrdg2a*) (see below). The deletion in Morex also led to the elimination of a LTR retrotransposon element belonging to the Copia/Ty1 family (Kumar and Bennetzen 1999). Automatic prediction of LTR by using the AutoPhredLTR tool of RiceGAAS allowed the identification of the LTRs between 27,257 to 26,937 bp and between 32,069 to 31,749 bp of HM124452. When the LTR sequences were compared, only one SNP was identified; assuming that at the time of insertion both LTRs were 100 % identical and considering a mutation rate of 6.5×10^{-9} substitutions per synonymous site per year (Gaut et al. 1996; SanMiguel et al. 1998), the approximate age of the insertion was estimated in about one million years ago.

Colinearity relationships in the two varieties are interrupted in the region between *Nbs2-Rdg2a* and *Nbs3-Rdg2a*. In this region a duplication of part of the *Nbs2-Rdg2a* LRR encoding domain (from base 38,341 to base 39,720 of HM124452) and a sequence inversion from base 45,294 to base 46,949 of HM124452 occurred in Morex. At position 26,156 of Morex contig, an *Nbs3-Rdg2a*-homolog sequence is present (*MNbs3-rdg2a*). Despite the high level of sequence identity, *MNbs3-rdg2a* carries a deletion of 2,705 bp with respect to Thibaut *Nbs3-Rdg2a* (*TNbs3-Rdg3a*), which eliminates the last 820 bp encoding the first LRR domain, the repetition of the NB domain and the first 533 bp of the second LRR encoding domain (Fig. 1; Bulgarelli et al. 2010).

Morex *rdg2a* arose from an apparent unequal recombination event

By considering the highest level of sequence similarity and informative polymorphisms highlighted from multiple alignment of *Rdg2a*, *Nbs2-Rdg2a* and *Mrdg2a* coding sequences (Figs. 2a, b, S1), the putative regulatory region and the first 555 bp of the coding sequence (a region that encompasses the CC domain and 108 bp encoding for the NB domain) of *Mrdg2a* are apparently derived from an *Rdg2a*-homolog sequence. From base 562 to the end of the transcribed region, homology relationships supported *Mrdg2a* derivation from an *Nbs2-Rdg2a*-homolog sequence (Figs. 2, S1). This is also confirmed by the presence, in *Mrdg2a*, of the same 201 bp deletion previously identified in the *Nbs2-Rdg2a* LRR encoding sequence with respect to *Rdg2a* (Bulgarelli et al. 2010; Fig. S1). These results support the hypothesis that *Mrdg2a* is derived from an intragenic unequal crossing over between ancestral *Rdg2a* and *Nbs2-Rdg2a* members of the gene family leading to the generation of a chimeric gene.

Mrdg2a encodes for a hypothetical CC-NB-LRR protein of 1,156 amino acids with an estimated molecular weight of 131.58 kDa (Fig. S2). Bioinformatic analyses using COILS software (http://www.ch.embnet.org/software/COILS) confirmed the presence of a CC domain from amino acid 25–60; while Pfam searches (http://pfam.sanger.ac.uk) showed a NB domain (from position 170 to 457) and a LRR (from amino acid 614 to 1,119) encoding sequence with *E* values of 2.1e−73 and 6.7e−05, respectively. As expected from nucleotide sequences, the comparison between MRDG2A, RDG2A and NB2-RDG2A protein sequences showed higher similarity between MRDG2A and NB2-RDG2A (89 % sequence similarity)

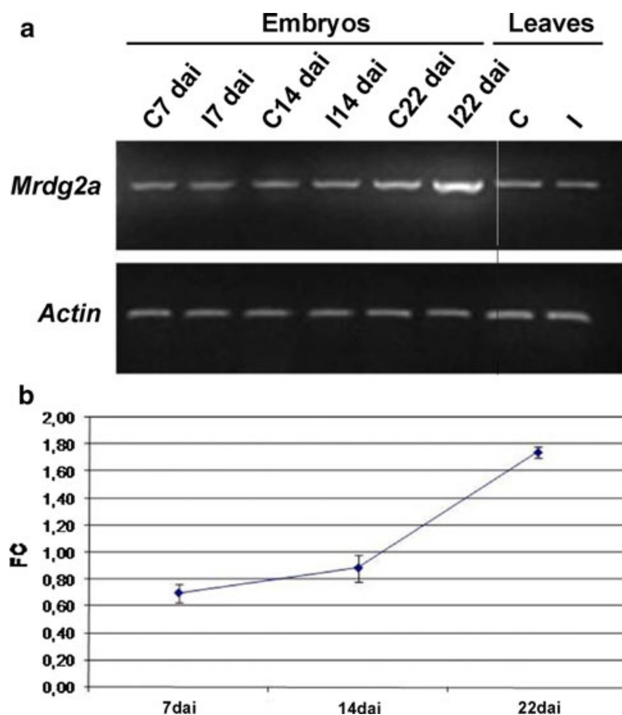


Fig. 3 **a** RT-PCR analysis of Morex *rdg2a* (*Mrdg2a*) under control condition (*C*) or after *P. graminea* isolate *Dg2* inoculation (*I*) at three time-points (7, 14 and 22 dai) in embryo and leaf tissues. Barley β *Actin* was used as an internal control. **b** Quantitative RT-PCR at 7, 14 and 22 dai for *Mrdg2a*. Values are expressed as Fold Change (FC) of transcript levels in inoculated embryos with respect to the transcript levels in control non-inoculated samples. Three technical replicates for two biological replicates were performed. *Error bars* represent standard deviation (SD) across all the technical replicates

than between MRDG2A and RDG2A (76 % sequence similarity). In particular, MRDG2A contained the same deletion of three LRRs, due to the 201 bp deletion, as NB2-RDG2A, with respect to RDG2A (Fig. S2).

Expression analyses performed by RT-PCRs showed that *Mrdg2a* is expressed in Morex embryos (the tissues where the fungal infection takes place) both in control conditions and after inoculation with *P. graminea* isolate *Dg2* at 7, 14 and 22 dai. The presence of *Mrdg2a* mRNA was detected in leaves of non-inoculated as well as infected 14-day-old Morex seedlings (Fig. 3a). qRT-PCR analyses of the time course experiment revealed that the expression of *Mrdg2a* did not change until 22 dai, when the transcription rate in the inoculated samples increased approximately 1.8 times with respect to control samples (Fig. 3b). In leaves, the mRNA accumulation was not pathogen-responsive.

To find out whether *Mrdg2a* conferred resistance to any *P. graminea* isolates, cv. Morex was infected with seven additional isolates of the pathogen. Increased resistance with respect to the susceptible control Mirco was detected for isolates *Dg19* (0 % infected plants) and *Dg9* (17 % infected plants) (Table 1). However, the role of *Mrdg2a* in increasing resistance against these two isolates should be demonstrated with transgenic approaches or through genetic mapping of *Mrdg2a* in a mapping population obtained by crossing Morex with Mirco, because it is not possible to rule out the implication of other genes than *Mrdg2a* in the observed resistant phenotype.

Table 1 Infection test with eight different leaf stripe isolates on nine barley genotypes

Barley genotype	Leaf stripe isolate							
	Dg2	Dg5	Dg1	Dg4	Dg9	Dg10	Dg12	Dg19
Thibaut	0*	100	0	66.5	0	0	0	0
Morex	95	76.5	100	95	16.5	30	45.5	0
Mirco	100	40.5	20.5	66.5	56.5	0	18.5	37.5
Golden promise	95	100	34.5	100	0	13.5	47.5	0
Acuario	0	100	0	43.5	0	0	0	0
Haruna nijo	0	100	0	66.5	0	0	0	0
Optic	0	96	0	65	0	0	0	0
Rebelle	0	0	0	50	0	0	0	0
Galleon	0	100	0	95.5	0	0	0	0

* For each interaction, the percentage of infected plants expressed as the means of three biological replicates is reported (LSD at $P < 0.05 = 0.77$)

High variability of the *Rdg2a* locus in different barley genetic backgrounds

To investigate whether the variability observed in the *Rdg2a* locus organization for Thibaut and Morex reflects the genetic distance between the two lines or if there is a general high variability in the cultivated barley gene pool, barley genotypes with resistant or susceptible reaction to isolates *Dg2* and *Dg5* was known, were chosen for a PCR-based molecular marker analysis of the genomic region (Table 2). The barley genotypes analyzed included *H. vulgare* ssp. *spontaneum* accession 41-1, that bears the *Rdg1a* resistance gene (Biselli et al. 2010), and four genotypes (Rebelle, Haruna Nijo, Galleon and Acuario) previously demonstrated carrying the *Rdg2a*-linked STS marker MWG2018 (Arru et al. 2003a). The analysis also included the *Dg2* and *Dg5*-susceptible variety Mirco which contains a non-functional *Rdg2a* allele (Bulgarelli et al. 2010).

The primer pairs for the analysis were designed in conserved (CR) and non-conserved (NCR) regions as defined from the comparison between Morex and Thibaut locus sequences (Fig. 1) and used to amplify genomic DNA from Thibaut, Morex and other 28 barley genotypes.

Table 2 Haplotype analysis at the *Rdg2a* locus in 30 barley genotypes

Cultivars	Dg2[a]	Dg5[a]	Haplotype	Primer combinations							
				CR1 CR2	NCR1 CR2	CR3 CR4	CR3 NCR2	NCR5 CR5	NCR3 CR6	CR7 CR8	NCR4 CR8
Thibaut	R	S	1	/	471	/	1,183	1,927	800	3,215	1,613
Morex	S	S	2	1,064	/	1,179	/	/	/	520	/
Optic	R	S	1	T[b]	T	T	T	T	T	T	T
Acuario	R	S	1	T	T	T	T	T	T	T	T
Galleon	R	S	1	T	T	T	T	T	T	T	T
Haruna Nijo	R	S	1	T	T	T	T	T	T	T	T
Rebelle	R	R	1	T	T	T	T	T	T	T	T
Imber	S	S	2	M[b]	M	M	M	M	M	M	M
Onice	R	R	3	T	M	T	1,250	M	M	M	M
Federal	R	R	4	T	200	T	1,670	M	T	T	T
H. vulgare ssp. *spontaneum* 41-1	R	R	5	T	300	T	1,660	M	M	M	M
Nudinka	S	S	6	T	200	T	1,680	M	M	T	T
Passport	S	S	6	T	200	T	1,680	M	M	T	T
Mirco	S	S	7	T	200	T	2,400	M	M	T	T
Rika	R	R	8	T	200	T	M	M	M	T	T
Bulbul	R	R	8	T	200	T	M	M	M	T	T
Proctor	R	R	8	T	200	T	M	M	M	T	T
Ansis	R	R	8	T	200	T	M	M	M	T	T
Alf	R	R	8	T	200	T	M	M	M	T	T
Diadem	R	R	8	T	200	T	M	M	M	T	T
Vada	R	R	8	T	200	T	M	M	M	T	T
Bonus	S	S	8	T	200	T	M	M	M	T	T
Ketos	S	S	8	T	200	T	M	M	M	T	T
Jaidor	S	S	8	T	200	T	M	M	M	T	T
Triumph	S	S	8	T	200	T	M	M	M	T	T
Golden Promise	S	S	8	T	200	T	M	M	M	T	T
Grete	S	S	8	T	200	T	M	M	M	T	T
Franka	S	S	8	T	200	T	M	M	M	T	T
Marado	S	S	8	T	200	T	M	M	M	T	T
Gitane	S	S	8	T	200	T	M	M	M	T	T

[a] Phenotypic response to leaf stripe isolates Dg2 and Dg5 inoculation: *R* resistant, *S* susceptible

[b] Haplotype at the *Rdg2a* locus: *T* Thibaut type, *M* Mirco type

The sizes of all amplification products as well as the absence of amplifications were in agreement with the proposed alignment of the *Rdg2a* locus in Morex and Thibaut (Fig. 1). PCR amplification with primers CR1 and CR2 produced a 1,064-bp long fragment in Morex whereas no amplification was obtained from Thibaut (Fig. 4) because of the indel of 4,829 bp (Fig. 1); while for primers NCR1 and CR2, a fragment of 471 bp for Thibaut and no amplification for Morex was detected. With the exception of cultivar Imber, which showed the same haplotype as Morex, for all the primer pairs tested, all other genotypes carried the Thibaut allele for the first primer combination (CR1–CR2; Table 2). For NCR1 and CR2, a 200-bp amplicon was observed for the majority of the cultivars, whereas *H. vulgare* ssp. *spontaneum* 41-1 yielded a 300-bp amplicon. Primers CR3 and CR4, flanking the large 13,692 bp deletion in Morex, amplified a 1,179-bp fragment in Morex and Imber only. All the genotypes carried the Thibaut haplotype, demonstrating that this deletion is not widespread among cultivated barley. CR3 and NCR2 amplified a 1,183 bp amplicon in Thibaut that included the *Rdg2a* promoter, whereas no amplification was observed in Morex. Five cvs. (Optic, Acuario, Galleon, Haruna Nijo and Rebelle), resistant to isolate *Dg2*, gave the same amplification as Thibaut, while fragments of bigger size were found for other genotypes (Federal, *H. vulgare* ssp.

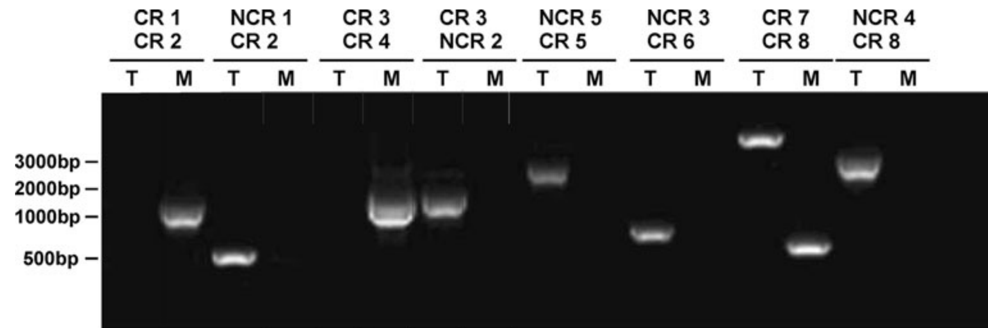


Fig. 4 PCR-based molecular marker analysis over the *Rdg2a* locus carried out on Thibaut genomic DNA (*T*) and Morex BAC 146G20 (*M*). Primer positions are indicated in Fig. 1

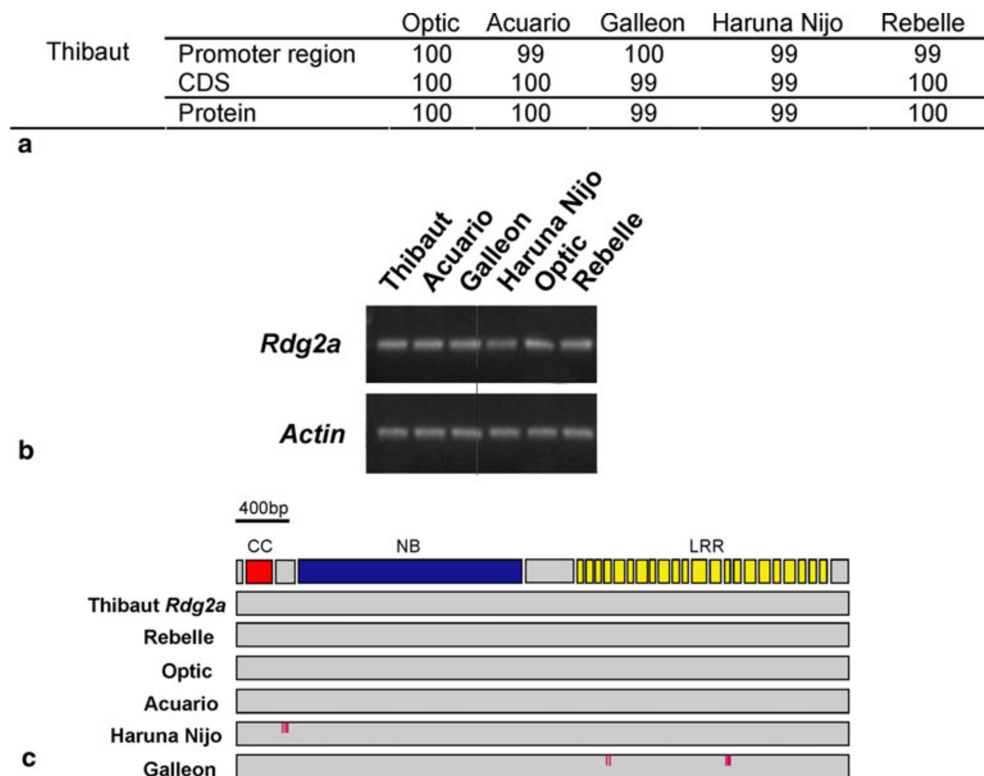
spontaneum, Nudinka, Passport and Mirco). Mirco *rdg2a* carries an insertion of 1,217 bp at the level of the promoter region (Bulgarelli et al. 2010) and, in agreement with this observation, this genotype showed a 2,400-bp long amplicon. Sizes of the PCR fragments of the other genotypes (Federal, *H. vulgare* ssp. *spontaneum* 41-1, Nudinka and Passport) suggested that, similarly to Mirco *rdg2a* (Bulgarelli et al. 2010), these alleles could belong to non-functional *Rdg2a* alleles carrying insertion in the promoter regions. According to this hypothesis, RT-PCR analyses showed that the *Rdg2a* alleles of these cultivars were not transcribed (data not shown).

Primer combination NCR5 + CR5 (1,927-bp long fragment in Thibaut and no amplification in Morex) yielded amplification products only in Optic, Acuario, Galleon, Haruna Nijo and Rebelle. For NCR3 and CR6, encompassing the *Nbs3-Rdg2a* promoter region, only Federal and the five cvs. carrying the Thibaut haplotype showed the same 800 bp amplicon like Thibaut. Finally, all the varieties, with the exception of Onice, Imber and *H. vulgare* ssp. *spontaneum 41-1*, showed Thibaut haplotype when PCRs were performed with primers annealing on the *Nbs3-Rdg2a* sequence (CR7 + CR8 and NCR4 + CR8). In conclusion, eight different haplotypes were identified, highlighting high variability in the cultivated barley gene pool. Interestingly, the four barley genotypes showing the same phenotype as Thibaut when challenged with isolates *Dg2* and *Dg5* (Optic, Acuario, Galleon and Haruna Nijo, resistant to *Dg2* and susceptible to *Dg5*; Table 1) showed the Thibaut haplotype for all the eight loci considered in this screening. Also Rebelle, resistant to both the isolates, carried the Thibaut haplotype. On the basis of these results it was possible to hypothesize that resistance to *Dg2* in these genotypes is mediated by *Rdg2a* alleles. Furthermore, as expected, all the *Dg2*-susceptible varieties analyzed showed a haplotype different from Thibaut; whereas for the *Dg2*-resistant cvs. with a haplotype different from Thibaut (i.e. Federal, *H. vulgare* ssp. *spontaneum* 41-1, Rica, Bulbul, Proctor, Ansis, Alf, Diadem, Vada), the presence of other leaf stripe resistance gene(s) different from *Rdg2a* should be hypothesized.

Rdg2a is highly conserved in different barley genetic backgrounds

Barley genotypes showing the same haplotype as Thibaut at the *Rdg2a* locus, namely Optic, Acuario, Galleon, Haruna Nijo and Rebelle, were selected for allele mining of the *Rdg2a* gene through allele re-sequencing. The genomic sequences of the alleles were obtained using PCR amplifications of overlapping fragments, including the promoter region, and direct sequencing of the amplicons. Blast2seq comparison of the promoter regions, the CDSs and the hypothetical encoded proteins of the *Rdg2a* alleles revealed an overall high level of sequence identity to Thibaut *Rdg2a* (Fig. 5a), suggesting that the new alleles identified might be functionally equivalent with respect to the *Rdg2a* allele of Thibaut. The promoter region of the Thibaut *Rdg2a* alleles was identical to each other with the exception of a SNP (C to A) 469 bp upstream the ATG for Acuario, Haruna Nijo and Rebelle. However, RT-PCRs demonstrated that all the five genes are expressed in embryos, underlying that this polymorphism did not affect the transcription process (Fig. 5b). The 99 % of identity with Galleon CDS was ascribable to two SNPs (both G to A) at 1,915 bp and 1,931 bp downstream the ATG, and a sequence change (ATGGT to TTAGG) from position 3,175 to 3,180 bp. These differences were predicted to result in the following amino acid conversions: Asp to Asn and Arg to Lys at positions 639 and 644 within the fourth LRR unit, Tyr to Met and Cys to Val at positions 1,059 and 1,058 in the 14 LRR (Fig. 5c). Haruna Nijo *Rdg2a* showed polymorphisms at 402 bp downstream the ATG (AGGA to GGAC) that resulted in two Arg instead of Lys-134 and Glu-135. These changes were located between the CC and the NB domains, at about 50 residues from the NB domain (Figs. 5c, S3, S4).

To find out whether the polymorphic nucleotides in Galleon and Haruna Nijo are subjected to positive selection, PAML 4 software (Yang 2007) was used to estimate the d_N/d_S (ω) ratio at each position of the CDSs alignment. The likelihood-ratio test (LRT) was employed comparing the ln-likelihood values (lnL) of the models of codon



Thibaut		Optic	Acuario	Galleon	Haruna Nijo	Rebelle
	Promoter region	100	99	100	99	99
	CDS	100	100	99	99	100
	Protein	100	100	99	99	100

Fig. 5 **a** Percentage of identity between the promoter regions, CDSs and protein products of Thibaut *Rdg2a* and Optic, Acuario, Galleon, Haruna Nijo and Rebelle homolog alleles. Blast2seq algorithm was utilized. **b** RT-PCR analysis of the *Rdg2a* alleles in Optic, Acuario, Galleon, Haruna Nijo and Rebelle genotypes. Thibaut genotype was used as control. Barley β *Actin* gene represents an internal control. **c** Schematic representation of the sequence alignment of the *Rdg2a* alleles identified in the allele mining analysis with Thibaut *Rdg2a*. The domains encoded by *Rdg2a* are indicated at the *top* (*red* CC; *blue* NB; *yellow* LRR; *grey* interspacer regions). *Red bars in the Rdg2a alleles* represent polymorphic nucleotides leading to non-synonymous changes in the protein and subjected to positive selection (color figure online)

substitution M7 and M8, which assume a β-distribution for ω ranging from 0 to 1, with M8 allowing also positive selected sites ($\omega > 1$) (Yang et al. 2000). The two models predicted a $\omega = 1$ for all the domains, due to the high level of sequence identity. As expected, M7 did not find any positive selected site, while M8 predicted the residues mutated in Galleon and Haruna Nijo under positive selection. In particular, this model estimated a posterior probability value of 0.952 for amino acid 134 and 0.966 for residues 639, 644 and 1,059 (Fig. 5c). Moreover, amino acids 135 and 1,058 were predicted to be under strong positive selection (posterior probability = 0.994 and 1, respectively). The likelihood ratios (2Δl) for comparing M8 to M7 were 18.32 and 8.27 for the LRR domain and the N-terminal region before the NB domain, respectively. Considering that these values are much greater than the corresponding χ^2 P value with degrees of freedom = 2 (0.00011 and 0.016, respectively), it was possible to conclude that M8 fitted the data significantly better than M7, giving confidence in considering the M8 predictions.

Positive selection is one of the major driving forces contributing to the generation of resistance specificities. Thus, to verify whether the identified positively selected amino acid conversions affected the resistance response to leaf stripe, Optic, Acuario, Galleon, Haruna Nijo and Rebelle were inoculated with six different isolates of the pathogen in order to identify possible differences in comparison with Thibaut resistance levels. 30 seeds for each variety were inoculated with each isolate in two biological replicates (Table 1). Rebelle provided the same pattern of resistance as Thibaut. Also Haruna Nijo showed the same phenotype with respect to Thibaut. The sequence differences between Haruna Nijo and Thibaut reside in the loop between the CC and the NB domains. It is well known that this region is not involved in the recognition of pathogen effectors (Lukasik and Takken 2009), thus, most likely, these mutations do not affect the leaf stripe resistance pattern of Haruna Nijo. The percentage of susceptibility to isolate *Dg4* increased to 95 % for Galleon with respect to other genotypes bearing the *Rdg2a* gene. As described above, Galleon carried four amino acids substitutions in the LRR domain that were, however, not localized in the β-strand/β-turn motif which determines the specificities of resistance (Dodds et al. 2001). Nonetheless, the observed increased level of susceptibility to one leaf stripe isolate raised the possibility that one or more of the observed amino acids changes affected the *Rdg2a* range of effectiveness toward leaf stripe.

Discussion

The *Rdg2a* locus contains three highly similar paralogous genes encoding for CC-NB-LRR proteins most likely resulting from a recent gene duplication followed by sequence diversification (Bulgarelli et al. 2010). The high level of sequence identity of the *Rdg2a* paralogs can likely promote sequence exchange through unequal crossing

over, as usually happens for plant loci bearing disease resistance gene analogs (Leister 2004). Comparison of the *Rdg2a* locus sequence in Thibaut (resistant) and Morex (susceptible) genotypes revealed rearrangements mainly consisting in three deletions which caused a difference in size of about 22.5 kbp in the two genotypes and a reduction of the number of genes at the locus from three to two. The biggest deletion of about 13 kbp was probably caused by an unequal crossing over between the ancestors of *Rdg2a* and its paralog *Nbs2-Rdg2a*. This event originated a hybrid gene deriving from an *Rdg2a* homolog sequence for the putative regulatory region and the first 562 bp of the coding sequence (including the CC domain and the first 555 bp of the NB domain) and from an *Nbs2-Rdg2a* homolog sequence for the rest of the CDS. Driving forces at the base of the evolution of plant disease resistance genes include unequal recombination between paralogs (Leister 2004) and gene conversion, as observed for the group A of *Pm3* alleles in wheat (Yahaiaoui et al. 2006). However, the presence of the relevant deletion of about 13 kbp in Morex rather supports unequal crossing over as the mechanism of Morex *rdg2a* origin. Examples of resistance genes derived from recombination events have been extensively reported in literature, among them the *Cladosporium fulvum* resistance gene *9DC* in tomato originated from unequal recombination between *Cf9* and its paralog *9DC* (Kruijt et al. 2004). More recently, Mirlohi et al. (2008) identified a hybrid gene generated from the barley stem rust resistance gene *Rpg1* and a closely linked paralog. The function of this gene has not been characterized but it seems to be involved in the recognition of a pathogen effector. Morex *rdg2a* appears to encode a complete CC-NB-LRR protein, is transcribed in both embryos and leaves in presence and absence of the fungus and is pathogen-responsive at 22 dai in infected embryos. Nonetheless, no clear resistance functions can be assigned because its resistance pattern is similar (or even more susceptible) to the reference leaf stripe susceptible genotype Mirco.

Based on the large differences observed at the *Rdg2a* locus for Thibaut and Morex, wide variability at this locus was expected in different resistant and susceptible barley genotypes. To mine the organization of the locus in barley, a PCR-based analysis was conducted at five different genomic regions spanning the *Rdg2a* locus highlighting eight different haplotypes. All the barley genotypes showing the same phenotype toward isolates *Dg2* and *Dg5* as Thibaut demonstrated to possess the Thibaut haplotype for all the tested amplicons. Morex haplotype was conserved only for the *Dg2* and *Dg5*-susceptible cv. Imber. The haplotype 8 was predominant, as detected in seven resistant and nine leaf stripe susceptible genotypes. For this haplotype, a Morex-type haplotype was observed for the primers' combinations designed in the *Rdg2a* and *Nbs2-Rdg2a* regions regardless of the level of leaf stripe resistance, underlying that these genotypes do not carry a functional *Rdg2a* homolog gene. Additionally, the observation that in several *Dg2*-resistant varieties, including *H. vulgare* ssp. *spontaneum 41-1*, a functional *Rdg2a* gene is absent (as supported by the haplotype analysis), indicates the presence of at least an additional leaf stripe resistance gene, different from *Rdg2a*, effective against *P. graminea* isolate *Dg2* in the barley gene pool.

The results of the haplotype analysis are in agreement with our previous observation that among the three paralogs identified at the *Rdg2a* locus only *Rdg2a* is effective against isolate *Dg2* (Bulgarelli et al. 2010). Primer combination NCR5 + CR5 (1,927-bp long fragment in Thibaut and no amplification in Morex) yielded amplification products only in Optic, Acuario, Galleon, Haruna Nijo and Rebelle, while no amplicons were obtained for the other leaf stripe-resistant genotypes confirming that *Nbs2-Rdg2a* is likely not involved in resistance.

To identify other functional alleles, *Rdg2a* was sequenced in the five *Dg2*-resistant genotypes showing the same Thibaut haplotype over the *Rdg2a* locus. A very high level of sequence identity was observed among the re-sequenced alleles. The only differences resided in two non-synonymous substitutions within the fourth and the fourteen LRRs for Galleon and three non-synonymous substitutions in the N-terminal domain, between the CC and the NB domains, in Haruna Nijo. Computational analyses showed that all the mutations identified were subjected to positive selection. Positive selection acting on the LRR domain, and in particular for the solvent-exposed residues of the β-sheet substructure, has been demonstrated being one of the major driving forces for the generation of new resistance specificities when there is a direct recognition between R and Avr proteins. In a recent study, Bhullar et al. (2010) identified, through a large scale allele mining, new alleles of the powdery mildew resistance gene *Pm3* in wheat and found that sequence variability was mostly located in the LRR encoding region. Similar results were also identified for the barley powdery mildew resistance locus *Mla* (Seeholzer et al. 2010) and the rice blast resistance locus *Pi2/9* (Zhou et al. 2007). Because positive selection at the level of the LRR domain was observed also among the three paralogs at the *Rdg2a* locus (Bulgarelli et al. 2010), we suppose that positive selection represents the major evolutionary force driving diversification at this locus in barley.

Evaluation of possible effects due to the positively selected variations identified in the *Rdg2a* paralogs highlighted a phenotypic effect only for Galleon, for which an increased susceptibility toward isolate *Dg4*, with respect to other *Rdg2a* alleles, was observed. This observation supports that amino acid changes in the Galleon *Rdg2a*-encoded protein could affect the range of effectiveness

toward leaf stripe. The variations detected in Haruna Nijo *Rdg2a* probably occurred at positions without a crucial role in resistance to *Dg2*, considering that no differences in response to *P. graminea* were observed. However, for both the cvs., it is not excluded that positive selection acting on the mutated residues could be responsible for an altered response to other not tested *P. graminea* isolates or other pathogens.

Although no studies have been performed to evaluate the frequency of leaf stripe resistance genes in *H. vulgare* ssp. *spontaneum*, this disease is typical of Mediterranean environments and leaf stripe is definitely present in the fertile crescent where *H. vulgare* ssp. *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. vulgare* ssp. *spontaneum* areas may have led to an increased frequency of leaf stripe resistance genes in the *H. vulgare* ssp. *spontaneum* gene pool. It is therefore expected that screening of *H. vulgare* ssp. *spontaneum* accessions could allow the identification of additional allelic variants. An *H. vulgare* ssp. *spontaneum* collection recently realized will allow additional phenotypic evaluations for leaf stripe resistance and suitable accessions will be subjected to haplotype and allele mining analyses with the purpose of identifying new *Rdg2a* alleles.

In conclusion, the present study provides evidences that rearrangements have shaped the *Rdg2a* locus during evolution and unequal recombination events contributed in generating different alleles. Even considering that no *Rdg2a* alleles with new specificities were identified, a pipeline for the identification of new *Rdg2a*-based leaf stripe resistance sources was established and information related to the role of substitutions and positive selection in the LRR encoding region as well as the identification of a phenotypic effect due to the substitutions for the barley genotype Galleon has proved to be useful for the successful isolation of *Rdg2a* alleles bearing new leaf stripe resistance specificities. Moreover, despite the small number of genotypes considered, we were able to identify new *P. graminea* isolate *Dg2*-resistant alleles among genotypes with different origin, thus increasing the small number of leaf stripe resistance sources that can be used in breeding programmes. Comparing the new alleles, sites under positive selection were identified and additional studies with larger collection of leaf stripe isolates will better clarify whether the new alleles can confer a broader resistance to leaf stripe.

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