Molecular analysis of fungal disease resistance in wheat

Keller B[1], Krattinger S[1], Yahiaoui N[1], Brunner S[1], Kaur N[1], Cloutre, C[1], Cloutier S[2], Ling HQ[3], Travella S[1]

[1]Institute of Plant Biology, University of Zurich, Zollikerstrasse 107, 8008 Zurich, Switzerland, [2]Cereal Research Centre, Agriculture and Agri-Food Canada, Winnipeg, MB R3T 2M9, [3] Institute of Genetics, Chinese Academy of Sciences, Beijing, China

ABSTRACT

Obligate biotrophic fungi such as rust species and mildew are major pathogens of wheat. Although a large number of resistance genes have been identified in the wheat germplasm against these pathogens, only few genes have been isolated at the molecular level. We have cloned the *Lr1* and *Lr10* leaf rust resistance genes and the *Pm3* alleles conferring powdery mildew resistance. For all these genes we have studied resistance gene evolution by analysing alleles/orthologs in diploid, tetraploid and hexaploid wheat species as well as grasses related to wheat. These studies have revealed highly diverse evolutionary histories of the different resistance genes, reflecting in part the human interference by agriculture. The most comprehensive study on natural diversity was undertaken for *Pm3* alleles in wild tetraploid wheat as well as a set of wheat landraces. This indicated an unexplored pool of additional resistance alleles particularly in landraces which are currently analysed for their agricultural use. Finally, we are analysing at the molecular level the functionally important domains in the Pm3 protein, allowing to perform the first experiments on molecular design of new resistance specificities.

INTRODUCTION

Plant pathogens are causing losses both by reducing the quantity of the harvested crop as well the quality of the product. It is estimated that in wheat, barley and rice around 10% of the total crop is destroyed by plant diseases. Yield losses reach 30% considering also pests and weeds (Oerke and Dehne 1997). Quality problems of the harvested product can be due to shrivelled seed which are frequently found as a consequence of the infection by leaf pathogens such as mildews, rusts, septoria, stagonospora blotch as well as many others. There also are direct quality problems caused by pathogens. In cereals, *Fusarium* species are the major culprit for mycotoxin contamination from the harvested grain, causing economic losses and in the worst case human and animal health problems.

Disease resistant plants are essential for sustainable agriculture. Although fungicides are available against many of the important cereal diseases, they are not affordable for farmers in many parts of the world, are sometimes difficult to apply because of weather conditions and can cause environmental problems. Historically, the applied goal of the genetic and later the molecular analysis of naturally occurring disease resistance has been to support and to improve resistance breeding in crop plants. However, the study of the classical and molecular genetics of disease resistance in plants has also been a scientifically rewarding area of basic research, revealing elementary mechanisms in the interactions of plants with pathogens at different levels. These studies have also resulted in the discovery of surprising similarities between pathogen defense in plants and defense mechanisms involved in innate immunity of animals and humans (Nuernberger et al. 2004).

Here, we will focus on the molecular analysis of the function, evolution and diversity of three disease resistance genes in wheat, which have been isolated in the last few years: *Lr10* (Feuillet et al. 2003), *Lr1* (Cloutier et al. 2007, Qiu et al. 2007) and *Pm3* (Yahiaoui et al. 2004, 2006; Srichumpa et al. 2005).

RESULTS AND DISCUSSION

Some of the important questions on wheat disease resistance genes concern their origin, their evolution and the availability of additional biological diversity in the gene pool which could be exploited for breeding. We have studied the molecular characteristics of three of the cloned fungal disease resistance genes in wheat: *Lr1*, *Lr10*, and *Pm3*.

The *Pm3* powdery mildew locus and its resistance alleles

There are seven naturally occuring resistance alleles at the *Pm3* locus which were all identified in modern wheat cultivars. These genes represent true alleles of the same gene and form an allelic series. The alleles are closely related at the sequence level and two groups of alleles can be distinguished: the alleles of one group are extremely similar to each other, with only a few amino acids differing from each other, possibly caused by point mutations (*Pm3d, e, g*). No silent mutations were observed among these alleles. The second group of alleles differ by polymorphic sequence blocks which are most likely the result of gene conversion events, either with homoeologuos genes or with paralogs from the gene cluster, of which *Pm3* is a member. The polymorphic blocks also contain silent nucleotide changes, indicating that their origin is relatively ancient (Figure 1).

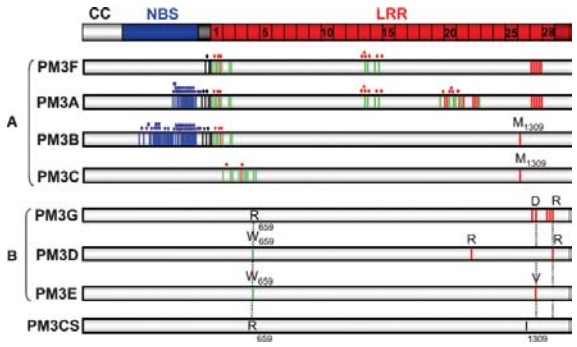

Figure 1: Schematic representation of protein sequences encoded by bread wheat *Pm3* alleles. Two groups of alleles (A and B) were distinguished based on their sequence divergence. The different domains encoded by *Pm3* genes are represented at the top: CC, coiled-coil; NBS (second box), nucleotide-binding site. The third box represents the spacer region between NBS and LRR domains. LRR, leucine-rich repeat. Leucine-rich repeat numbers are indicated. Bars in the PM3 resistance proteins represent polymorphic amino acids compared with the PM3CS protein, also representing the consensus sequence of the resistance alleles. Dots represent synonymous mutations. Dashed vertical lines indicate identical positions of amino acid changes in the group B proteins (from Yahiaoui et al. 2006).

From the sequence of the seven alleles, a consensus sequence was derived. Interestingly, this sequence is found at the *Pm3* locus of many powdery mildew susceptible wheat lines, among them the cv. Chinese Spring. Therefore, we call it *Pm3CS* (for Chinese Spring, but also for consensus sequence). In transient transformation assays of leaf epidermal cells, this allele resulted in a susceptible interaction, demonstrating that it represents a susceptible allele. The most parsimonious interpretation of these data favors a model of gene evolution in which all the modern, active resistance alleles of *Pm3* are derived from an ancient template sequence represented by the *Pm3CS* gene. This gene was either never an active resistance gene or, more likely, is a gene which was once active but was later overcome because it was present in a wide variety of wheat material.

To further understand the evolutionary history of the *Pm3* resistance alleles, we analysed the *Pm3* locus in tetraploid wheat, both in accessions of wild *T. turgidum* ssp. *dicoccoides* as well as in cultivars/breeding lines of domesticated tetraploid wheat. None of the seven functional *Pm3* alleles from hexaploid wheat was identified in more than 200 tetraploid lines. However, the susceptible *Pm3CS* allele was found in several wild wheat accessions. They were all geographically found in the South Eastern part of Turkey from the region of the Karacadag Mountains. Interestingly, the same geographic area had been identified in an earlier study to be the region of origin of domesticated tetraploid wheat (Ozkan et al. 2005).

The sequencing of 4kb of 5' upstream sequences in all seven *Pm3* resistance alleles as well as *Pm3CS* revealed completely identical sequences and an estimate of the maximal evolutionary age of this upstream sequence is less than 3000 years. All these observations can be used to formulate a model on *Pm3* gene evolution as follows: The *Pm3CS* gene was present in a wild ancestor of tetraploid wheat which was domesticated in Turkey. Possibly, if was at that time an active resistance gene and we speculate that avirulent races might still exist for this gene at least on tetraploid wheat. Such a domesticated tetraploid wheat was involved in the formation of hexaploid wheat where the *Pm3CS* allele became widespread, resulting in strong selection on the pathogen population for loss of avirulence. Finally, based on the *Pm3CS* template sequence, gene conversion as well as point mutation events created the active *Pm3* resistance alleles which are currently used in the wheat breeding pool. Thus, the *Pm3* – powdery mildew pathosystem would be evolutionary very young and represent a rare example fitting the arms race model for host pathogen evolution.

The wheat-powdery mildew interaction can be easily studied in a transient system. There, a gene construct of interest is bombarded by a particle gun into the epidermis of the wheat leaf, together with a reporter gene construct. A few hours later, the leaves are infected with a defined powdery mildew isolate. After 48 hours, the transformed cells are identified by staining for reporter gene activity, and these cells are then analyzed for a compatible interaction (as defined by haustoria formation) or an incompatible interaction (arrest of fungal growth at the appressoria stage). By analyzing three independent experiments and >100 cells, a haustorial index can be determined. We are using this system to test new forms of chimeric resistance alleles of *Pm3* based on the variation in the existing alleles to identify the molecular basis of specificity and activity. We have found that the susceptible *Pm3CS* allele can be converted by a single base pair change into a resistant allele, demonstrating that the derivation of new resistance alleles from this susceptible template is relatively straightforward. We are now using this system to study the molecular basis of *Pm3* function in more detail.

To analyze different aspects of *Pm3* function in the whole plant, transgenic wheat lines for all seven *Pm3* alleles have been made. These transgenic lines contain modified genes which are under control of the strong *ubi* promoter from maize, and we want to study the consequences of different expression levels on the plant. In addition, the constructs contain an HA-tag for later possibility to detect biochemically the Pm3 resistance proteins.

Evolution of the wheat *Lr10* leaf rust disease resistance locus

The leaf rust resistance gene *Lr10* was isolated (Feuillet et al. 2003) using a combination of subgenome map-based cloning and haplotype studies. *Lr10* is closely associated with a second resistance gene analog (*RGA2*), but the two genes are very different from each other at the molecular level. Analysis of the wheat gene pool by Southern hybridization revealed the presence of two groups of lines with distinct haplotypes (H1 and H2) at the *Lr10* locus, defined by the presence (H1) or absence (H2) of the two full-length *Lr10* and *RGA2* genes on chromosome 1A. Thus, the *Lr10* resistance locus differs from most resistance loci in plants, where clusters of closely related genes are present, but is similar to the *Arabidopsis RPM1* locus (Grant et al. 1995). The *RPM1* locus also exists in two stable haplotypes in the *Arabidopsis* gene pool that are defined by the presence or absence of the gene.

We found that at the molecular level the two haplotypes differ by a large deletion/inversion event which converted the H1 haplotype to H2. Interestingly, both haplotypes are ancient and can be found in diploid, tetraploid and hexaploid wheat (Isidore et al. 2005, Figure 2), with the same deletion event as the basis of haplotype 2. This evolutionary old situation contrasts strongly from the observation at the *Pm3* locus and indicates a mechanism of balancing selection at the *Lr10* locus. There, both haplotypes are evolutionary conserved over long periods of time, possibly because the presence of the *Lr10* gene does not only give an advantage in resistance, but comes with some cost to the plant. The deletion/inversion events in the H2 haplotype had several consequences for the further evolution of the *Lr10-RGA2* region in the wheat gene pool. In fact, it may have effectively inhibited recombination between the different haplotypes. If recombination occurs within the inverted sequence, the recombinant gametes will carry chromosomes that are partially deleted or duplicated. Such gametes probably have a greatly reduced fitness, and recombination between the haplotypes in the *Lr10-RGA2* interval is effectively suppressed. This provides a molecular explanation for two earlier observations; first, there are no recombinant haplotypes between H1 and H2 in the gene pool, and the *Lr10* gene was always found together with an intact *RGA2*. Second, there was no recombination between the two genes in a segregating population of more than 6000 gametes originating from a cross between the cultivars Frisal and Thatcher*Lr10*, which belong to the H2 and H1 haplotypes, respectively. Thus, our data indicate that recombination in hexaploid wheat cannot only be blocked by chromosomal segments derived from recent introgressions from wild relatives, but also by inversions that originate from ancient haplotypes

We have recently made an extensive study of the tetraploid wheat gene pool for the diversity present at the *Lr10* locus. We found in some tetraploid wheat lines genes identical to the *Lr10* leaf rust resistance gene of hexaploid wheat lines containing *Lr10* (C. Loutre, unpublished data). This demonstrates that *Lr10* is derived from tetraploid wheat, in contrast to the situation of *Pm3* where none of the active resistance alleles in hexaploid wheat could be found in the tetraploid gene pool.

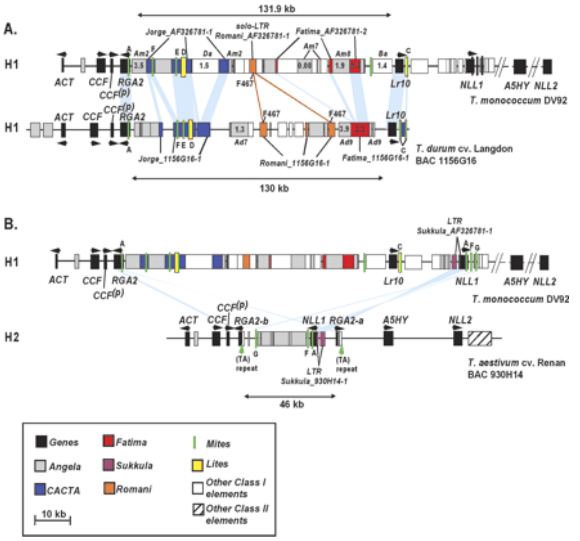

Figure 2: Schematic representation of the sequence organization and detailed comparison at the *Lr10* orthologous loci in diploid, tetraploid, and hexaploid wheat. (*A*.) Organization of the orthologous loci in *T. monococcum* and *T. turgidum* and comparison of the genomic region between *RGA2* and *Lr10* (haplotype H1). (*B*.) Organization of the orthologous loci in *T. monococcum* and *T. aestivum* and comparison between *T. monococcum* (H1) and *T. aestivum* (H2) in a region delimited by the two *RGA2* fragments of the *T. aestivum* sequence. Light-grey areas indicate conservation between the orthologous regions in the *RGA2–Lr10* interval. The genes are indicated by black boxes. The short arrows located *above* or *below* the genes indicate the transcriptional orientation. For more details see Isidore et al. (2005).

The evolutionary origin of the *Lr1* leaf rust disease resistance gene

The third fungal disease resistance gene that we have studied is the *Lr1* leaf rust resistance gene cloned by Cloutier et al. (2007). It encodes a CC-NBS-LRR type of protein of 1344 amino acids. We have found in an earlier screen of more than 200 *Ae. tauschii* (goat grass, D-genome donor) accessions that approximately 3.5% of these accessions displayed the same low infection type against an isolate of leaf rust avirulent on *Lr1* as the tester line Thatcher *Lr1*. The accession Tr.t. 213, which showed resistance after artificial infection with *Lr1* isolates both in Mexico and in Switzerland, was chosen for further analysis. Genetic analysis showed that the resistance in this accession is controlled by a single dominant gene, which mapped at the same chromosomal

position as *Lr1* in wheat. These results indicated that the resistance gene in *Ae. tauschii* accession Tr.t. 213 is an ortholog of the leaf rust resistance gene *Lr1* of bread wheat, suggesting that *Lr1* originally evolved in diploid goat grass and was introgressed into the wheat D genome during or after domestication of hexaploid wheat.

We have now studied the allelic diversity of the *Lr1* gene in different wheat cultivars and in two *Aegilops tauschii* accessions differing in a leaf rust resistance gene mapping at the *Lr1* locus (Qiu et al. 2007). The *Lr1* ortholog in *Ae. tauschii* line 213 was highly similar to the bread wheat *Lr1*, whereas *Lr1* differed significantly from its alleles in susceptible bread wheat lines or the ortholog in *Ae. tauschii* line 1704. In particular, the two resistant alleles share a block of 605 bp in the LRR encoding region (Figure 3). This block was possibly derived from a recombination or gene conversion event, and it is likely that it forms the molecular basis of *Lr1* resistance activity. There are a few more, non-diagnostic polymorphisms in other regions of the protein, but they are unlikely to be involved in the difference between resistant and susceptible alleles. We conclude that a very close and functional *Lr1* gene was already present in the diploid gene pool of *Ae. tauschii* which was then introgressed into bread wheat.

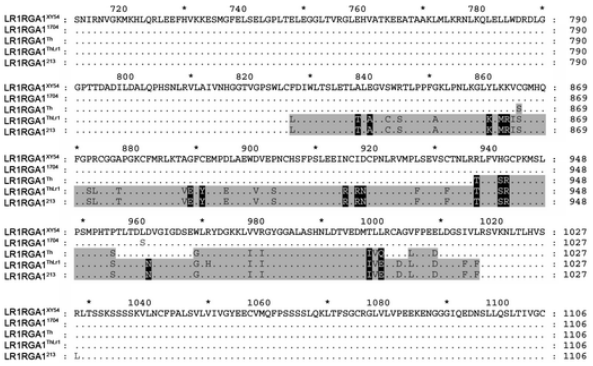

Figure 3: Partial alignment of the LRR domain encoded by susceptible (*LR1RGA*^{XY54}, *LR1RGA1*^{1704}, and *LR1RGA1*Th) and resistant (*LR1RGA1*^{ThLr1} and *LR1RGA1*^{213}) alleles of *Lr1* from bread wheat and *Ae. tauschii* (alleles 213 and 1704). The comparison of amino acid sequences encoded by susceptible and resistant *LR1RGA1* alleles identifies polymorphic sequence blocks in the LRR region (amino acids 827–1,016) that are possibly resulting from recombination/gene conversion events. *Dots* represent identical amino acids. *Grey shaded areas* represent the blocks of sequences indicative of recombination/gene conversion events. *Black shaded* amino acids within these sequence blocks are polymorphic solvent-exposed amino acids (x residues in the LxxLxLxx motif in LRR repeats). For more details see Qiu et al. (2007).

OUTLOOK

The molecular and functional analysis of three wheat genes conferring resistance to fungal pathogens has revealed different evolutionary histories. For two genes, we could show that the resistance gene was already present in an ancestor of bread wheat, whereas for *Pm3* there is good evidence that it evolved only after formation of hexaploid wheat.

Given the detected characteristics of resistance evolution as well the discoveries on the molecular basis of resistance function, it should be possible in the longterm to artificially create new functional specifities of wheat *R* genes. However, there is a clear lack of knowledge on protein 3-D structure to allow such strategies at the current state. Finally, it will be important to learn more on the fungal part of the interaction, in particular on the avirulence genes involved in the interaction with the specific *R* genes.

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