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*Biotechnol. Agron. Soc. Environ.* 2002 **6** (2), 79–85

# Amplified fragment length polymorphism (AFLP) analysis of markers associated with *H5* and *H22* Hessian fly resistance genes in bread wheat

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Received 25 February 2002, accepted 12 April 2002.

Hessian fly, *Mayetiola destructor* (Say), is one of the most destructive pest of wheat (*Triticum* species) worldwide. In Morocco, damage caused by Hessian fly can result in total crop loss if high infestations occur during early stages of crop development. Genes that confer resistance to Hessian fly provide the most efficient and economical means of crop protection against this damaging insect. To date, 27 resistance genes (*H1–H27*) have been reported in wheat; among these, 11 are very effective in Morocco. In this study, we have utilized amplified fragment length polymorphism (AFLP) analysis in conjunction with near-isogenic lines (NILs) and bulked segregant analysis (BSA) to identify molecular markers linked to Hessian fly resistance genes in bread wheat. Two pairs of NILs were used as source of DNA, one differing for *H5* resistance gene and the other for *H22* resistance gene. Using 42 primers combinations, 4200 selectively DNA fragments were analyzed throughout the wheat genome, with an average of 100 bands per combination and per pair of NILs. This technique appeared to be promising, since 28 polymorphic bands were detected, among which 13 associated to *H5* locus and 15 to *H22* locus.
**Keywords.** *Mayetiola destructor*, *Triticum*, *H5* gene, *H22* gene, amplified fragment length polymorphism (AFLP), near-isogenic lines (NILs), bulked segregant analysis (BSA), pest resistance, Morocco.

**Analyse parAFLP de marqueurs associés aux gènes *H5* et *H22* de résistance à la mouche de Hesse chez le blé tendre.** La mouche de Hesse, *Mayetiola destructor* (Say), est un insecte qui, au niveau mondial, cause des dégâts considérables chez le blé (*Triticum* spp.) dans la majorité des aires de production. Au Maroc, ces dégâts peuvent aller jusqu'à une perte totale du rendement si des niveaux d'infestation élevés coïncident avec le stade jeune de la plante. L'utilisation de cultivars résistants reste la méthode la plus efficace et la plus économique pour le contrôle de ce ravageur. Vingt-sept gènes de résistance désignés *H1* à *H27* ont été répertoriés et parmi ceux-ci, 11 se sont montrés très efficaces contre les populations marocaines de l'insecte. Dans cette étude, nous nous sommes proposés de chercher des marqueurs moléculaires liés aux gènes *H5* et *H22* de résistance à la mouche de Hesse chez le blé tendre. À cette fin, deux paires de lignées presque isogéniques de blé tendre dont chacune est constituée par des lignées qui ne diffèrent que par l'absence/présence du segment du chromosome portant le locus du gène étudié (*H5* ou *H22*) ont été utilisées. Ces lignées ont été analysées par la technique d'AFLP en combinaison avec la méthode d'analyse de ségrégation en mélange (BSA) qui facilite la détection des marqueurs adjacents au locus cible. Ainsi, en testant 42 combinaisons d'amorces, 4200 loci ont été analysés, à raison de 100 bandes en moyenne par combinaison et par paire de lignées isogéniques. Vingt-huit loci polymorphes ont été détectés dont 13 sont liés au segment chromosomique portant le gène *H5*, et 15 au segment portant le gène *H22*.
**Mots-clés.** *Mayetiola destructor*, *Triticum*, gène *H5*, gène *H22*, AFLP, lignées isogéniques, BSA, résistance aux organismes nuisibles, Maroc.

## 1. INTRODUCTION

Hessian fly, *Mayetiola destructor* (Say), is a major insect pest of wheat (*Triticum* species) throughout most production areas of the world. In Morocco, Hessian fly losses were estimated respectively at 36% and 32% for bread wheat (*Triticum aestivum* L.) and durum wheat (*T. durum* Desf.) (Amri *et al.*, 1992a; Lhaloui *et al.*, 1992). Moreover, high damages, even total crop losses can be observed if high infestations occur at early developmental stages (Amri *et al.*, 1992a). The use of genetic resistance to this pest is actually the most effective control and constitutes an economical and an environmental sound approach. Up to now, 27 resistance genes (*H1–H27*) have been identified in wheat as effective against this pest in USA (McIntosh, 1988; Patterson *et al.*, 1988; Cox, Hatchett, 1994; Ohm *et al.*, 1997). In Morocco, the wheat resistance genes *H5*, *H7H8*, *H11*, *H13*, *H14H15*, *H21*, *H22*, *H23*, *H25* and *H26* are effective against Hessian fly (Gallagher *et al.*, 1987; El Bouhssini *et al.*, 1988, 1998; Amri *et al.*, 1990, 1992b). Most of these major resistance genes have been incorporated into adapted Moroccan bread wheat (Jlibene, 1992; Jlibene *et al.*, 1993).

The primary resistance mechanism is antibiosis, where young larvae initiating feeding on resistant plants are killed by natural plant substances (Gallun *et al.*, 1975). A gene-for-gene relationship was demonstrated for host resistance and insect avirulence loci (Hatchett, Gallun, 1970), but biochemical resistance mechanisms are unknown. Although the optimum strategy for gene deployment has to be elaborated, the single gene strategy has been adopted successfully. However, as the biological interactions between wheat (*Triticum* spp.) and Hessian fly (*Mayetiola destructor*) are highly specific, the widespread use of resistant cultivars exerts a strong selection pressure on the Hessian fly populations. This favors new virulent biotypes (strains) capable of surviving and reproducing on resistant wheat plants (Gallun *et al.*, 1975). Therefore, entomologists and plant breeders have to identify continually new sources of resistance genes to replace those that are no longer effective and to properly deploy existing genes in order to increase their durability.

Molecular markers are becoming essential tools in plant breeding (Staub *et al.*, 1996; Mohan *et al.*, 1997; Gupta *et al.*, 1999) and have several advantages over the traditional phenotypic markers that are difficult or time-consuming to select by plant breeders. These DNA type markers are not influenced by environmental conditions and are detectable at all plant growth stages. Availability of tightly linked molecular markers can now be used in marker-assisted selection (MAS) programs, specially for disease resistance gene where it is possible to infer the gene by the marker without depending on the natural pest or pathogen occurrence or waiting for its phenotypic expression. Moreover, molecular markers flanking disease resistance genes may be starting points for genes cloning and subsequently comprehension of their biological mechanisms (Martin *et al.*, 1993; Tanksley *et al.*, 1995).

Restriction fragment length polymorphism (RFLP) markers have been routinely previously used for agronomic crops linkage analysis and genomes mapping (Tanksley *et al.*, 1989). However, construction of RFLP maps has been very difficult due to the low level of polymorphism in a self-pollinated crop such as wheat (Chao *et al.*, 1989). With the development of polymerase chain reaction (PCR) technology, some alternative strategies for generating molecular markers such as random amplified polymorphic DNA (RAPDs) (Welsh, McClelland, 1990; Williams *et al.*, 1990), sequence tagged sites (STS) (Inoue *et al.*, 1994), microsatellites (Röder *et al.*, 1998) and amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995) have emerged. Because AFLP technique permits inspection of polymorphism at a large number of loci distributed throughout a plant genome, within a very short period of time and requires very small amount of DNA, it provides new opportunities for mapping and gene tagging in plants with large genomes and low polymorphism rates such as wheat (Breyne *et al.*, 1997; Gupta *et al.*, 1999; Harlt *et al.*, 1999; Ridout, Donini, 1999).

In this study, we present the application of AFLP technique using near-isogenic wheat lines (NILs) and bulked segregant analysis (BSA). As a first step of exploiting the utility of AFLP in the wheat genome mapping program, and eventually for marker-assisted breeding, we studied the polymorphism in two pairs of NILs that differed for *H5* and *H22* genes which confer resistance against Moroccan Hessian fly biotypes. We combined the AFLP technology as a strategy able to screen a high number of loci, with the BSA approach which facilitates the detection of markers adjacent to the target loci, in order to quickly identify linked markers to genes of interest. The simultaneous use of both BSA and NILs methods reduces the risks of false positives. NILs and/or BSA combined with different molecular technologies have been successfully used to tag several disease resistance genes in different economically important plants (reviewed by Lefebvre and Chèvre, 1995; Staub *et al.*, 1996; Breyne *et al.*, 1997; Gupta *et al.*, 1999).

## 2. MATERIALAND METHODS

### 2.1. Plant materials

Plant materials used in this study consisted of two pairs of bread wheat near-isogenic lines (NILs) (Jlibene, 1996). The first pair includes NILs differing

only in the chromosome 1A region containing the *H5* loci and the second includes NILs differing only in the chromosome 1D region containing the *H22* loci. These NILs were produced by four repeated backcrossing of an F1 hybrid with the susceptible parent. For the latter, two Moroccan varieties (Marchouch and Kanz) were used; the *H5* gene was transferred from Saada line to Marchouch and *H22* gene from KS85WGRC01 line to Kanz.

### 2.2. DNA extraction and BSA

Wheat genomic DNA was isolated from resistant and susceptible near-isogenic lines and from parental lines (Saada, Kanz, Marchouh and KS85WGRC01). Leaf tissue (2 g) was ground in liquid nitrogen and suspended in 20 ml of extraction buffer (*100 mM NaCl, 50 mM EDTA pH 8, 2% SDS, 100 mM Tris-HCl pH 8.0, 0.1 mg.ml$^{-1}$ Proteinase K*). The homogenate was incubated in water bath at 60°C for 1 h. The lysate was extracted with an equal volume of phenol/ chloroform and the aqueous fraction mixed with an equal volume of isopropanol. Precipitated DNA was removed from solution, washed in 70% ethanol, dissolved in TE buffer (*10 mM Tris-HCl, 1mM EDTA, pH 8*) and treated with RNAse (25 µg·ml$^{-1}$) for 1 h at 37°C. Finally, the DNAwas precipitated with absolute ethanol and 3 M sodium acetate and re-suspended in TE buffer. The DNA concentration was determined on spectrophotometer.

Resistant and susceptible DNA bulks (250 ng.µl$^{-1}$) were prepared by pooling equal amounts of genomic DNA samples from respectively ten susceptible and ten resistant individual NILs for each gene population.

### 2.3. AFLP analysis

The AFLP protocol (Vos *et al.*, 1995) was followed with some modifications. The restriction reaction was carried out with 500 ng genomic DNAof each pool or parent to which was added 5 units of SseI and MseI enzymes and 8 µl of 5 X RL buffer (*50 mM Tris acetate pH 7.5, 50 mM magnesium acetate, 50 mM potassium acetate, 25 mM DTT, 250 ng·µl$^{-1}$ BSA*) in a final reaction volume of 40 µl and incubated at 37°C for 3 h. After complete digestion, 10 µl of solution containing 50 pMol MseI adapter, 5 pMol SseI adapter, 1mM ATP, 1 unit $T_4$ DNA ligase and 2 µl 5 X RL buffer were added to the restriction fragments and incubated for 4 h at 37°C. Ligated DNA template was diluted 10-fold with sterile $TE_{0.1}$ (*10 mM Tris-HCl, 0.1 mM EDTA, pH 8*). The ligation product (5 µl) was amplified in 40 µl PCR reaction volume containing 200 µM of each dNTP, 0.5 units of Taq DNA polymerase, 4 µl of 10 X PCR buffer (*100 mM Tris pH 8.3, 15 mM $MgCl_2$, 500 mM KCl*) and 75 ng of each MseI and SseI primer without any additional selective nucleotide at the 3' end. The PCR pre-amplification profile was 30 cycles of 30 s at 94°C, 30 s at 56°C and 1 min at 72°C, followed by final extension at 72°C for 5 min. The PCR product was then diluted 10-fold with sterile $TE_{0.1}$. Selective amplification was conducted with two or three selective bases at the 3' end of both primers. The SseI selective primer was end-labeled with $^{32}$P-γATP before amplification. The selective amplification profile was 1 cycle of 30 s at 94°C, 30 s at 65°C and 1 min at 72°C, followed by lowering the annealing temperature, each cycle of 0.7°C for twelve cycles, followed by 23 cycles at an annealing temperature of 56°C. Amplified products were resolved by electrophoresis on 4.5% denaturing polyacrylamide gel. The gel was then dried and exposed to X-ray film for 16 h. Only clear and unambiguous bands were scored.

## 3. RESULTS

A total of 42 MseI/SseI primer combinations was used to test parents and bulks (see material and methods). By labeling the SseI primer and using 2 or 3 bp extension on both primers, we typically observed on average 100 unambiguous selectively amplified DNA fragments on the autoradiograph from any given primer pair ranging from approximately 50 to 500 base pairs (bp). Polymorphic fragments were distributed across the entire size range. Assuming that each AFLP band corresponds to a genetic locus, we estimated that 4200 loci were screened from each parental genome and for each gene. The polymorphic bands or specific AFLP markers were identified as bands present in the resistant parent and bulk but missing in susceptible parent and bulk. Specific bands detected in the susceptible parent were not analyzed. Examples of AFLP patterns showing two markers associated to *H5* gene and three to *H22* gene are given in **figure 1** and **figure 2** respectively. Among the 28 polymorphic fragments detected, 13 markers were found in the target segment chromosome carrying the *H5* locus and 15 associated to *H22* locus. All markers were linked in coupling phase to *H5* and *H22* resistance alleles (linked with the allele conferring resistance).

A list of the primer pairs and their numerical success rates (numbers of polymorphic fragments identified) is given in **table 1**.

Out of 42 primer combinations that were tested, 9 generated polymorphic fragments between samples differing by the presence/absence of *H5* gene, whereas 12 gave polymorphic bands for NILs bulks and parents corresponding to *H22* gene and four were shared between the two sets of NILs (**Table 1**).

The number of polymorphic fragments was low for each pair and varied from one band to three. The low

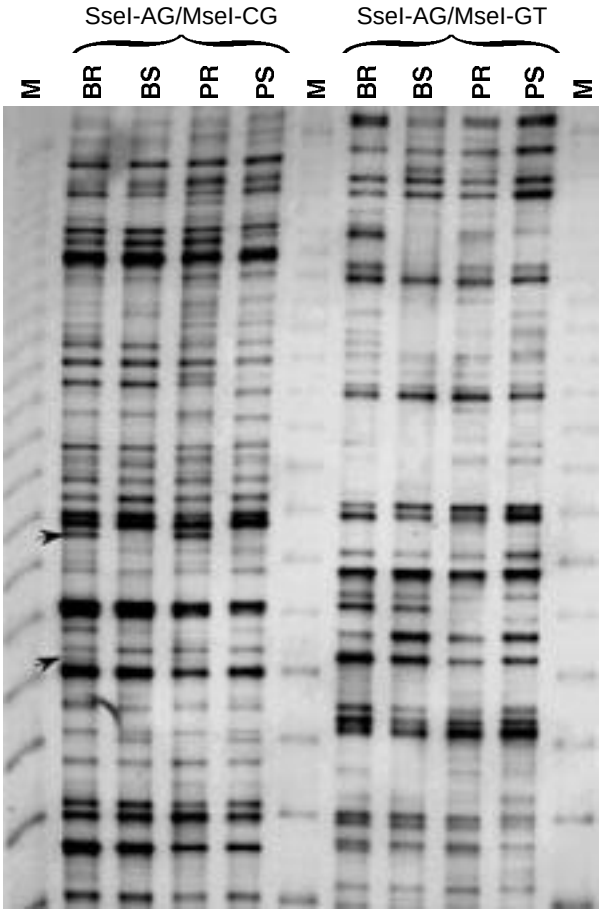


**Figure 1.** AFLP pattern showing two polymorphic markers associated to *H5* gene resistance. Polymorphic bands are pointed with an arrow — *Profil d'AFLP montrant deux bandes polymorphes associées au gène de résistance* H5. *Les bandes polymorphes sont indiquées par une flèche* BR = resistant NILs bulk — *bulk résistant*; BS = susceptible NILs bulk — *bulk sensible*; PR = resistant parent (Saada) — *parent résistant (Saada)*; PS = susceptible parent (Marchouch) — *parent sensible (Marchouch)*; M = 30-330 bp molecular weight marker — *marqueur de taille moléculaire 30-330 pb*.

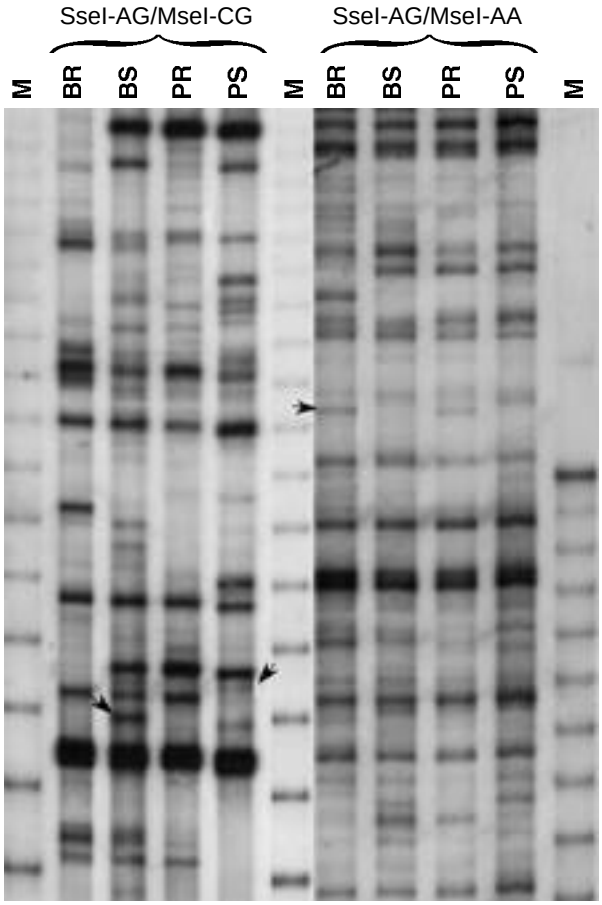


**Figure 2.** AFLP pattern showing three polymorphic markers associated to *H22* gene resistance. Polymorphic bands are pointed with an arrow — *Profil d'AFLPmontrant trois bandes polymorphes associées au gène de résistance* H22. *Les bandes polymorphes sont indiquées par une flèche* BR = resistant NILs bulk — *bulk résistant*; BS = susceptible NILs bulk — *bulk sensible*; PR = resistant parent (KS85WGRC01) — *parent résistant (KS85WGRC01)*; PS = susceptible parent (Kanz) — *parent sensible (Kanz)*; M = 30-330 bp molecular weight marker — *marqueur de taille moléculaire 30-330 pb*.

polymorphism confirms that between the NILs analyzed and the recurrent parent only a small percentage of the genome is different.

## 4. DISCUSSION

Depending on the size of the genome to be analyzed, different sets of primers will have to be used. In our study, MseI- SseI- digested genomic DNAs from pools and parents were used as template for selective PCR amplification with MseI and SseI primers. Our choice of restriction enzymes and primer sequences was based on preliminary screenings which detected the different primers exhibiting a maximum number of bands on acrylamide gels (results not showed). In this study we explored the possibility of using AFLP markers for the detection of polymorphism in wheat. This AFLP technique has been used to identify markers linked to disease resistance genes (Thomas *et al.*, 1995; Harlt *et al*, 1999) and assess genetic diversity in several important agronomic crops including wheat (Breyne *et al.*, 1997; Gupta *et al.*, 1999).

Moreover, the use of NILs is based on the concept that the DNAs of the recurrent parent and its NIL are mostly identical except in a small portion of the donor genome which contained the introgressed gene (Muehlbauer *et al.*, 1988). In principle, polymorphic

**Table 1.** Combinations of SseI/MseI primers used for selective amplification AFLP and numbers of polymorphic fragments identified in Hessian fly resistant bulk/parents for *H5* and *H22* resistance genes — *Nombre de bandes polymorphes identifiées pour chacune des 42 combinaisons d'amorces SseI/MseI utilisées, lors de l'amplification sélective des DNA des lignées isogéniques pour le gène* H5 *et pour le gène* H22.

| **MseI Primer** | **SseI Primer** | | | | | |
|---|---|---|---|---|---|---|
| | **+AC** | | **+AG** | | **+CA** | |
| | **H5** | **H22** | **H5** | **H22** | **H5** | **H22** |
| **+ AA** | 0 | 2 | 0 | 0 | 0 | 1 |
| **+ AG** | 1 | 0 | 0 | 0 | 0 | 0 |
| **+ CA** | 1 | 0 | 0 | 1 | 1 | 1 |
| **+ CG** | 0 | 0 | 2 | 2 | 0 | 1 |
| **+ CT** | 0 | 0 | 0 | 0 | 0 | 0 |
| **+ GA** | 0 | 0 | 0 | 0 | 0 | 0 |
| **+ GC** | 1 | 1 | 1 | 0 | 0 | 1 |
| **+ GG** | 0 | 0 | 0 | 0 | 0 | 0 |
| **+ GT** | 3 | 1 | 0 | 0 | 0 | 0 |
| **+ TA** | 0 | 0 | 1 | 0 | 0 | 0 |
| **+ TC** | 0 | 0 | 0 | 0 | 0 | 0 |
| **+ACG** | 0 | 0 | 0 | 0 | 0 | 1 |
| **+AGA** | 0 | 1 | 0 | 0 | 0 | 0 |
| **+ CAT** | 2 | 0 | 0 | 0 | 0 | 2 |
| **Total** | 8 | 5 | 4 | 3 | 1 | 7 |

Numbers indicate the polymorphic fragments observed.

genetic markers between the NIL and its recurrent parents are potentially linked to introgressed gene. To facilitate the detection of markers in the genetic background of the NILs, phenotypic pools may successfully be applied to characterize monogenic traits, such as Hessian fly resistance genes. Thus, we used bulked segregant analysis (BSA) which allow to study genomic region of interest against a randomized genetic background of unlinked loci (Michelmore *et al.*, 1991). With this approach, DNA samples from susceptible and resistant plants are bulked separately. Within each pool, the mixed individuals are identical for the gene of interest but arbitrary for all other genes.

At present, some Hessian fly resistance genes were tagged with RFLP or RAPD molecular markers in wheat. Ma *et al.* (1993) and Delaney *et al.* (1995) identified RFLP markers linked to *H23*, *H24* and *H25* Hessian fly resistance genes, whereas with the help of NILs, 13 Hessian fly resistance genes were marked with RAPDs technology (Dweikat *et al*., 1994, 1997; Seo *et al.*, 1997). However, the RFLP analysis has some limitations as time-consuming and labor-intensive and RAPD is known to suffer from a lack of reproducibility. AFLP is a marker system able to detect high levels of polymorphism and uses stringent annealing conditions which guarantee a better reproducibility. Using 42 AFLP primer pairs, we surveyed 4200 loci throughout the wheat genome for each gene population and observed 13 polymorphic fragments linked to *H5* locus and 15 to *H22* locus that are located on chromosome 1 AS and 1 D respectively (Roberts, Gallun, 1984; Raupp *et al*., 1993). The low level of polymorphism detected in the current study was anticipated because NILs and their recurrent parents are very closely related and share over 95% of their genomes in common (Jlibene, 1996).

The present study, to our knowledge, is the first to apply AFLP technique to identify polymorphism associated to resistance gene against Hessian fly biotypes in wheat. However, the study of segregating populations remains a prerequisite to determine among the markers identified those that are tightly linked to the pest resistance genes and to determine exact location and genetic distance. The linked markers can be used for indirect selection and pyramiding genes resistance in wheat as they allow the fast screening of large numbers of plants without subjecting them to insects in early stages of development (Liu *et al.*, 2000). Moreover, the expression of Hessian fly resistance genes is influenced by environmental variables such as temperature (Sosa, 1979; Tyler, Hatchett, 1983). Thus, molecular markers linked to resistance genes allow the simultaneously screening of multiple markers without the limitation of environmental factors.

In order to facilitate the analysis of AFLP markers identified in large populations of individual plants, we will attempt to convert the linked AFLP markers to SCAR-PCR markers. This procedure called characterized amplified region (SCAR) (Paran, Michelmore, 1993; Thomas *et al.* 1995; Harlt *et al*. 1999) can be more easily employed with large populations using a single and cheap PCR test that can be of use in MAS and for pyramiding Hessian fly resistance genes.

## 5. CONCLUSIONS

In this investigation, we show that the AFLPtechnique combined with BSA and NILs can play an important role in cereal improvement programs as it is effective in polymorphism identification between very tightly related lines. To confirm genetic linkage between the markers detected and the respective gene, a F2 population of plants segregating for each individual gene is currently being screened. The combination of different resistance genes in new wheat cultivars by means of both conventional and molecular based breeding methods, will be required to improve the durability of cultivars resistance in the future. To prevent a rapid breakdown of *H5* and *H22* once they should be integrated into new wheat varieties,

additional Hessian fly genes resistance have to be combined. In order to pyramid several genes in the same variety, markers flanking all these genes are needed. Future studies should be directed to detect markers for additional Hessian fly resistance genes that are still effective.

**Acknowledgement**

The authors thank D. Mingeot for reading the manuscript; R. Baleux and L. Kutten for technical assistance. This work is supported by an AUPELF/UREF grant (Action de Recherche Partagée), a postdoctoral fellowship from AUPELF/UREF to Bouchra Najimi (bourse d'excellence) and CIERSACO Project.

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