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Characterization of *Pyrenophora graminea* Markers Associated with a Locus Conferring Virulence on Barley

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The fungus *Pyrenophora graminea* is the causal agent of barley leaf stripe disease. Two leaf stripe isolates PgSy3 (exhibiting high virulence on the barley cultivar 'Arabi Abiad') and PgSy1 (exhibiting low virulence on Arabi Abiad), were mated and 63 progeny were isolated and phenotyped for the reaction on Arabi Abiad. The population segregated in a 1:1 ratio, 32 virulent to 31 avirulent (χ^2 = 0.05, *P* = 0.36), indicating single gene control of PgSy3 virulence on Arabi Abiad. Among 96 AFLP markers identified, three AFLP markers, E37M50-400, E35M59-100 and E38M47-800 were linked to the virulence locus *VHv1* in isolate PgSy3. The results of this study indicate that (the three markers) are closely linked to *VHv1* and are unique to isolates carrying the virulence locus. This work represents an initial step towards map-based cloning of *VHv1* in *P. graminea*.

Keywords : AFLP markers, barley, leaf stripe, *Pyrenophora graminea*, virulence gene

Barley leaf stripe caused by the Ascomycete fungus *Pyrenophora graminea* [anamorph *Drechslera graminea* (Rabenh. *ex* Schltdl.) Ito], is one of the most widely distributed seed-borne diseases of barley (*Hordeum vulgare* L.) (Tekauz, 1983; Mathre, 1997). The development of methods for producing sexual crosses of the fungus in culture (Smedegaard-Petersen, 1987) has facilitated conventional genetic studies on the inheritance of pathogenicity and virulence. The culture conditions that favor the hybridization between two opposite mating types of this pathogen were established by Arabi and Jawhar (2007).

Barley resistance to *P. graminea* has been reported in both quantitative (Arru et al., 2003; Pecchioni et al., 1996) and qualitative forms (Skou et al., 1994; Tacconi et al., 2001). The presence of qualitative resistance suggests the potential for a gene for gene interaction occurring in this pathosystem. Although *P. graminea* causes an economically devastating disease and a fair amount of research has been done on host resistance, few studies specifically investigating the genetics conditioning virulence have been conducted.

Several pathotype studies have focused on the variability of virulence in *P. graminea* in Wild populations (Gatti et al., 1992; Jawhar et al., 2000). Based on the resistance genes present in the differential sets used, a high level of variability was identified in worldwide collections of *P. graminea* indicating that many different virulence factors are present in the population. These factors could be either virulence genes used to induce infection, avirulence genes recognized by the host to induce resistance or a combination of these mechanisms. There is much to be learned about the genetics of virulence and the cause of the various observed phenotypes in *P. graminea*, as few such studies have been conducted. However, identification of virulence factors of *P. graminea* is important for the fundamental understanding of infection and leaf stripe progress in barley and for the development of control strategies (Arabi et al., 2005). In a previous study, genetic analysis showed that a single locus (now designated *VHv1*) in the most Syrian virulent isolate PgSy3 controls high virulence on barley (Arabi and Jawhar, 2007).

Little is known about the genetic and molecular interactions of *P. graminea* with barley. To fully elucidate these interactions, it is important to catalog, isolate, and characterize genes for virulence or pathogenicity in the pathogen and genes for resistance in the host. Amplified fragment length polymorphism (AFLP) has been used to construct genetic linkage maps in fungi (Vos et al., 1995). Due to its simplicity, stability and polymorphism detection frequency, AFLP has been a popular tool for constructing recombination based genetic linkage maps in fungi (Lin et al., 2006; Lind et al., 2007). In this study, a fungal population generated from a cross of *P. graminea* isolates that showed deferential disease reactions on multiple barley genotypes was used for the genetic characterization of a virulence locus (*VHv1*) in *P. graminea*

Fungal isolates. After an extensive screening for over ten years in the greenhouse and in the laboratory, the two

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isolates PgSy1 and PgSy3 were proved to be the most avirulent and virulent genotypes on all barley cultivars available so far. Therefore, they were selected in this study. *P. graminea* cultures used for virulence evaluation initially were established from single spores collected from infected barley leaves. Dry leaves infected with *P. graminea* were surface sterilized in 5% sodium hypochlorite (NaOCl) for 5 min. After three washes with sterile distilled water, the pieces were transferred onto Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) with 13 mg/l kanamycin sulphate added after autoclaving and incubated for 10 days, at 22 ± 1 ºC in the dark to allow mycelial growth and sporulation.

***P. graminea* mating.** Barley straw was cut into 3–4 cm in length. The tissue was autoclaved at 18 psi for 30 min and transferred to Petri dishes containing agar media with ~ 1 cm separating each straw piece. Conidia were scraped (with scalpel) from plates after adding sterile water, and the suspension was adjusted to 10.000 conidia per ml. A 0.1 ml aliquot of the suspension of each isolate was pipetted onto the straw pieces to initiate mating. Plates were incubated at 15℃ (Weiland et al., 1999). After 4 weeks, several pseuduothecia were removed at weekly intervals and crushed under a cover glass for microscopic examination. When mature ascospores were found, single pseuduothecia were individually removed to agar blocks (1 cm^2), which were adhered to the lid of a Petri plate containing water agar. After replacing the lid, the covered dish was incubated under fluorescent light at room temperature. Over the course of several hours, ascospores were ejected from asci within the pseuduothecia down onto the water agar. By turning the lid every 15 min, the separation of individual ascospores on the agar surface was achieved. Ascospores were transferred to PDA media for the production of conidia that were subsequently used for virulence testing.

Virulence phenotyping. The inoculation experiments were performed on cv. Arabi Abiad from Syria. The *P. graminea* parent and progeny isolates were grown in Petri dishes on PDA medium at 22 ± 1℃ for 10 days in the dark. Barley seeds were inoculated using the method described by Hammouda (1986). Samples of the seeds were first checked with a refrigerated blotter test (Porta-Puglia et al., 1986) to ensure that they were not naturally infected. A randomized complete block design with three replications of 10 pots of five plants each was used for each experiment. Following the inoculation technique, 50 seeds for each isolate were surface sterilized in 70% ethanol for 30 sec and 5% sodium hypochlorite (NaOCl) for 5 min, rinsed well in three changes of deionized water and then incubated in Petri dishes containing an actively growing mycelium cultured on PDA medium. After 14 days of incubation in the dark at 6℃, the emerged seedlings were transplanted into 12-cm diameter pots and grown in the greenhouse at 12 ℃/10 h dark and 20℃/14 h light. At the heading stage (GS 50) (Zadoks et al., 1974) plants were examined for leaf stripe symptoms and were classified as diseased (reduced size and leaves with long necrotic stripes) or healthy (normal size and absence of stripes). The incidence of the disease was expressed as percentage of infected plants using a scale of 1–5 (Delogu et al. 1989). No plants showing leaf stripe symptoms were found among the non-inoculated controls. The experiments were repeated twice for each isolate.

DNA extraction and bulk segregant analysis. DNA extraction was performed according to Aljanabi and Martinez (1997). Bulk segregant analysis (BSA) (Michelmore et al., 1991) was carried out with 63 progeny derived from the cross PgSy1 and PgSy3. Equal concentrations of DNA from 32 virulent progeny were bulked into three pools. Three similar pools also were constructed using DNA from 31 avirulent ones. These six pools then were used to identify AFLP markers linked to the avirulence gene. Any markers found linked to the pools were screened against the entire mapping population.

AFLP assay. AFLP was carried out using a method modified from Vos et al. (1995), with 250 ng of DNA used as the starting template. Primers containing only one selective base (E-A, E-C, E-G, M-A, M-C, or M-T) were used for the preselective amplification. In all, 12 *Eco*RI primers containing two selective bases (E-AA, E-AC, E-AG, E-AT, E-CA, E-CC, E-CG, E-CT, E-GEA, E-GC, E-GG, or E-GT) and 12 *Mse*I primers containing two selective bases (M-AA, M-AC, M-AG, M-AT, M-CA, M-CC, MCG, M-CT, M-TA, M-TC, M-TG, or M-TT) were used in all combinations for selective amplification reactions. Primer pairs showing a large number of polymorphisms between parental isolates were used to screen the bulked DNA pools. AFLP bands were run on 6% polyacrylamide gels and visualized by silver staining. Markers were named using the four extension letters from the selective primers and the band size.

Data analysis and linkage group construction. All AFLP markers detected between parental isolates PgSy1 and PgSy3 were evaluated on 63 progeny isolates and tested for deviations from an expected 1:1 segregation ratio using the χ^2 test ($\alpha = 0.05$). For the purposes of mapping, virulence ratings similar to the avirulent parent and markers originating from this parent were scored as 'a' while virulence similar to the highly virulent parent and markers originating from this parent were scored as 'b.' Linkage analysis was

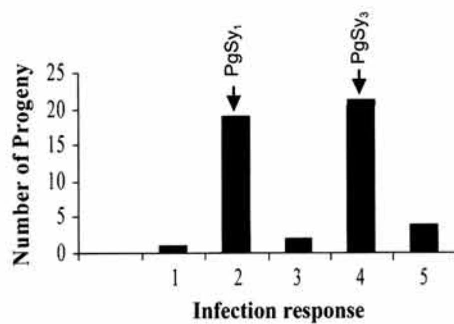

Fig. 1. Disease incidence score on the barley cv. 'Arabi Abiad' by *Pyrenophora graminea* progeny of the mapping population derived from the $PgSy_1$ and $PgSy_3$ cross. The 1 to 5 scale along the horizontal axis is based on the incidence scale of Delogu et al. (1989). The parental infection response is indicated by arrows.

performed using the computer program MAPMAKER version 2.0 (Lander et al., 1987). Linkage between ordered loci was calculated using the Kosambi mapping function (1944). To determine the critical LOD threshold, a permutation test consisting of 5000 permutations was executed. A LOD threshold of about 2.8 in this population yields an experiment-wise significance level of 0.05.

The cross between $PgSy_1$ and $PgSy_3$ was selected to generate a mapping population to identify a virulence gene. These parental isolates were selected because of their mating compatibility, the large number of ascospores produced from the cross, and their contrasting virulence on the barley genotype Arabi Abiad. In all, 74 single ascospore progeny were originally isolated from this cross, but this number was reduced to 67 after some ascospores failed to germinate and others were identified as clones (via identical AFLP banding patterns).

Progeny isolates that produced infection responses (IRs) in the 1–2 range were considered to possess low virulence, whereas those that produced IRs in the ≥ 3 range were considered to possess high virulence. Many of the progeny exhibited the same IR exhibited by the parent isolates (Fig. 1). The population segregated 32:31 virulent/avirulent ($\chi^2 = 0.05$, $P = 0.36$) when phenotyped for virulence on cv. Arabi abiad, suggesting single gene control of the virulent phenotype. This conclusion is also based on the assumption that the mechanism of ascospore abortion was nonselective for one or the other virulence genotype. Similar inheritance pattern was reported in the progeny of crosses among *P. teres* f. *teres* (Weiland et al., 1999). On the other hand, the reaction levels demonstrated in the cv. Arabi Abiad is in close agreement with those reported by Arabi et al. (2005). They reported that the aggressive isolate $PgSy_3$ had a direct impact on storage proteins in the latter barley cultivar, whereas, no such effect was noticed when $PgSy_1$ was used.

Parental isolates PgSy1 and PgSy3 were screened with 42 AFLP primer pairs producing an average of 50 bands per reaction and 3.6 polymorphisms between the parents. Twenty-three primer pairs were selected based on the high number of scorable polymorphic bands produced and the diversity of selective bases in the primers. Three AFLP markers (E37M50-400, E35M59-100 and E38M47-800) co-segregated with *VHv1* at a distance of 14.1, 5.2 and 17.1 cM, respectively (Fig. 2) were identified by BSA as linked to the virulence phenotype. However, the distribution of these markers among virulent and avirulent *P. graminea* isolates were different, the percentage of existing these markers were high in the virulent isolates (Table 1). It is well known that the genetic distance between two points corresponds to crossing over frequency, and thus, to new recombination possibilities (Vale et al., 1994; Paterson, 1996). According to that, we can consider the marker E35M59-100 to be the closest to one single locus controlling virulence trait in *P. graminea*. Regarding these results, we can say that the presence of the three positive (virulent)

Table 1. AFLP markers associated with barley specific virulence locus VHv1 in the *P. graminea* F1 mapping population (N=63) derived from a cross between isolates PgSy1 and PgSy3

Virulence phenotypes	AFLP Markers	% Isolates
Virulent	E37M50-400	34.38
	E35M59-100	23.44
	E38M47-800	37.50
Avirulent	E37M50-400	9.38
	E35M59-100	4.69
	E38M47-800	9.38

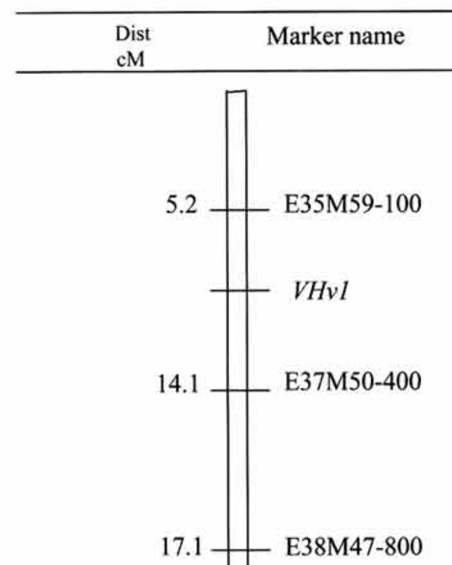

Fig. 2. Linkage group with AFLP markers associated with the barley cultivar-specific virulence locus *VHv1* in *P. graminea*.

alleles is more authentic in marker-assisted selection for virulence character in *P. graminea*.

Although the number of markers identified in this study was sufficient to identify linkage groups containing major virulence genes, more markers are needed to saturate this map. Future genomics studies will focus on the saturation of this map with more markers as well as the correlation of linkage groups with physical chromosomes. *P. graminea* is a haploid fungus thus it is impossible to characterize dominance. Therefore, these genes could code for virulence gene products used by the pathogen to exploit the host or they could code for avirulence gene products that are recognized by the host to signal a resistance response. Either way these genes are important in the host pathogen interaction and further characterization of these genes is needed. The construction of a linkage group and identification of closely linked DNA markers provide a solid starting point for the positional cloning of *VHv1* in *P. graminea.*

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