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Isolate and organ-specific QTLs for ascochyta blight resistance in faba bean (*Vicia faba* L).

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Abstract The main objective of the present study was to locate the genomic regions responsible for ascochyta blight resistance in faba bean. Six QTLs were identified with the help of a linkage map constructed from a F_2 population from the cross between the inbred lines 29H (resistant) and VF136 (susceptible). Two pathogenically distinct *Ascochyta* isolates were used to study the genetic control against them and disease evaluations were performed separately on leaves and stems to investigate whether different genetic systems control resistance in each plant organ, as previously suggested. The six QTLs detected were named *Af3* to *Af8*. *Af3* and *Af4* were effective against both *Ascochyta* isolates, *Af5* was only effective against isolate CO99-01 while *Af6*, *Af7* and *Af8* were only effective against isolate LO98-01. *Af3*, *Af4*, *Af5* and *Af7* were revealed in both leaves and stems. By contrast, *Af6* was only effective in leaves and *Af8* only in stems. The validity and application of these results in a MAS program is discussed.

Introduction

Ascochyta fabae Speg. is the causal agent of ascochyta blight in faba bean (*Vicia faba* L.). It is a common disease that can cause yield losses of 35 to 40%, which can reach up to 90% in susceptible cultivars when the environmental conditions are favourable for disease development (Hanounik 1980). The symptoms caused by *A. fabae* develop on leaves, stems and pods, and in severe pod infections even seeds can be infected. Since *A. fabae* is a seed-borne pathogen, infected seeds are the main source of inoculum, although crop debris and volunteer plants may also have a role in pathogen spreading. Moreover, the teleomorph, *Dydimella fabae* Jellis and Punith; could also affect the disease cycle by producing ascospores that can be wind-dispersed over greater distances, increasing genetic diversity in the pathogen and adversely affecting disease management.

Disease control through crop rotation, clean seeds and chemical treatment has not been completely effective, and the development of resistant cultivars is widely recognized as the most efficient method of control. Since Bond and Pope (1980) and Kharbanda and Bernier (1980) reported differences in disease incidence among cultivars, several sources of resistance to *A. fabae* have been identified in different backgrounds (Ali and Bernier 1985; Jellis et al. 1985; Tivoli et al. 1987; Hanounik and Robertson 1989; Pritchard et al. 1989; Rashid et al. 1991a; Sillero et al. 2001). However, breeding efforts to achieve complete resistance to *A. fabae* have been unsuccessful because of the complexity of the trait and the lack of detailed knowledge about the loci involved in its control. There is limited and confusing information available about the genetic basis of resistance. Genetic studies on resistance to *A. fabae* in faba bean have shown both polygenic and major gene inheritance. Thus, Rashid et al. (1991a) suggested seven single genes controlling the resistance to isolates of *A. fabae*. Hanounik and Robertson (1989) and Kohpina et al. (2000) found two types of resistance: one narrow-based specific resistance and a broad-based general resistance. Roman et al. (2003) reported polygenic control determined by at least two quantitative trait loci (QTLs) in their mapping population. In addition, resistance on leaf and stem has been suggested to be under different genetic control (Rashid

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et al. 1991b; Kharrat et al. 1997; Kohpina et al. 2000), although further research is needed to establish whether differences in scores on both organs have a genetic basis or are determined by environmental conditions (Kohpina et al. 2000).

Physiological specialization between pathogen isolates and host genotypes has also been described (Kharbanda and Bernier 1980; Hanounik and Robertson 1989; Rashid et al. 1991b; Kohpina et al. 1999). This differential interaction leads to the necessity of including several resistance genes in a breeding program to avoid overcoming of resistance (Bond et al. 1994), or of using genotypes with a wide resistance spectrum against different isolates of the pathogen (Hanounik and Robertson 1989; Bond et al. 1994). In addition to this, the evaluation of resistance is sometimes hampered by environmental conditions, which interfere with the evaluation process (Melchinger 1990; Michelmore 1995; Mohan et al. 1997).

Taking into account all the factors described above, complexity of the resistance and simultaneous analysis of different isolates and plant organs, greatly complicates the phenotypical identification of resistance in any breeding program.

Over the past years, molecular markers have been identified for genes/QTLs controlling resistance to several biotic stresses in legumes, such as soybean (Kim and Diers 2000; Arahana et al. 2001; Li et al. 2002; Yuan et al. 2002), bean (Miklas et al. 2001; Park et al. 2001; Schneider et al. 2001; Fall et al. 2002), lentil (Ford et al. 1999), chickpea (Santra et al. 2000) or faba bean (Roman et al. 2002, 2003; Avila et al. 2003b). The identification of molecular markers tightly linked to the *A. fabae* resistance genes may provide a valuable alternative to the time-consuming disease evaluation. Moreover, indirect selection would facilitate stabilization of genetic resistance through gene pyramiding. Recently, two QTLs for partial resistance to *A. fabae* against one isolate have been identified in a F_2 population from a cross between Vf 6 and Vf 136 (Roman et al. 2003). These QTLs acted in an additive fashion and all the resistance alleles were derived from the partially resistant parent Vf 6. Nevertheless, the authors did not address the question of the isolate-specificity of this resistance or the possible genetic basis for the differences in disease scores from leaves and stems. Therefore, the objectives of this work were: (1) to locate the genomic regions responsible for quantitative resistance in the faba bean-ascochyta blight pathosystem using a new parental line (29H) as source of resistance, and (2) to investigate to what extent these QTLs are isolate or organ-specific in their action.

Materials and methods

Plant material

Resistance studies and molecular analyses were carried out using the F_2 plants and $F_{2:3}$ families from the cross between the faba bean inbred lines 29H and Vf 136. The maternal parent 29H was provided by Drs. Berthelem and Le Guen (Station d'Amélioration des Plantes, INRA-Rennes, France), and has been described as resistant to *A. fabae* in several studies (Tivoli et al. 1987; Maurin and Tivoli 1992; Bond et al. 1994; Sillero et al. 2001). The male parent Vf 136 belongs to the germplasm collection of the C.I.F.A. (Centro de Investigación y Formación Agraria) "Alameda del Obispo" in Córdoba (Spain), and has been described previously as susceptible to ascochyta blight (Roman et al. 2003).

Resistance scoring

One hundred and fifty nine $F_{2:3}$ families were tested for ascochyta blight resistance under growth chamber conditions. Ten plants per $F_{2:3}$ family were inoculated with each isolate. Plants were grown at 20°C in 2-l pots (five plants per pot), using a 1:1 mixture of sand and peat. Both parents and the susceptible check, VS-172, were included in all the evaluations. Seedlings were inoculated when the third leaf was fully expanded, by spraying a suspension of *A. fabae* spores prepared with tap water (5×10^5 conidia/ml), to which 0.03% (v:v) Tween-20 was added. Two virulent monoconidial isolates of *A. fabae*, CO99-01 and LO98-01, originating from Córdoba (Southern Spain) and Logroño (Northern Spain), respectively, were used. Plants were incubated during 48 h in the dark at 100% relative humidity, and then kept at 20°C, under a photoperiod of 14 h light and 10 h dark, with light intensity of 148 μmol/m^2/s at the leaf canopy. Fifteen days after inoculation, disease resistance was scored separately on leaves and stems. On leaves two criteria were used: (1) the 0–5 "infection type" scale (IT) (Rashid et al. 1991b) based on the lesion type where 0 = no sign of infection and 5 = large coalescing lesions >5 mm in diameter with pycnidia; (2) "disease severity on leaves" (DSL), an estimation of the percentage of symptomatic leaf area. On stems, only the second criterion: "disease severity on stems" (DSS) was used representing the percentage of symptomatic stem area. Assessments were made as the average of the scores in ten plants per family and per isolate.

Molecular analysis

The variability of the parental lines was tested for eight isoenzymatic systems: aspartate aminotransferase (AAT; EC 2.6.1.1), aconitase (ACO; EC 4.2.1.3), alcohol dehydrogenase (ADH; EC 1.1.1.1), esterase (EST; EC 3.1.1), fructokinase (FK; EC 2.7.1.4), glucose-6-phosphate isomerase (GPI; EC 5.3.1.9), phosphogluconate dehydrogenase (PGD; EC 1.1.1.44), peroxidase (PRX, E.C. 1.11.1.7) and superoxide dismutase (SOD, E.C. 1.15.1.1). Systems showing polymorphism were tested in the F_2 population. Horizontal starch-gel electrophoresis was performed on 12% starch gels (Gottllieb 1973). All the assay solutions were adapted from those reported by Wendel and Weeden (1990) except for FK (Muehlbauer et al. 1989). Results on the genetics and chromosome location, obtained for each isozyme locus, are reported elsewhere (Torres et al. 1998; Avila et al. 2003a).

Random Amplified Polymorphic DNA (RAPD) analysis was performed as described (Welsh and McClelland 1990; Williams et al. 1990) with modifications (Torres et al. 1993). A total of 420 primers from Operon Technologies (Alameda, California, USA) were surveyed in the parental lines. Primers, revealing intense and clearly scorable polymorphic bands, were screened on the F_2 population for linkage analysis.

The cross was also tested for five seed-protein genes (USP, Vicilin and legumins A, B3 and B4). Sequences and characteristics of the primers used for detection of length polymorphism among these genes were previously reported (Macas et al. 1993a) and amplification conditions were as described by Vaz Patto et al. (1999).

Nine microsatellite markers (GA-4, GAII-8, GAII-30, GAII-54, GAII-59, GAII-68, GATA2, GATA11 and JF1-AG3), physically located on chromosome 1 of *V. faba* (Pozarkova et al. 2002), were analyzed in the parental lines. Those revealing polymorphisms were tested in the segregant population.

Linkage analysis

Each marker was tested against the expected segregation ratio using a chi-square goodness of fit in the F_2 population. The linkage map was constructed by MAPMAKER/EXP version 3.0 (Whitehead Institute, Cambridge, Massachusetts, USA; Lincoln et al. 1993). A LOD threshold of 3 was established to consider significant linkage. Recombination fractions were converted to centiMorgans (cM) using the Kosambi's mapping function (Kosambi 1944).

QTL analysis

The linkage map and the quantitative data of resistance against *A. fabae* were used for the QTL analyses. QTL location was performed using three different methods included in the QTL CARTOGRAPHER software (Basten et al. 2001): Linear regression (LR, in LRmapqtl), Simple Interval Mapping (SIM, Model 3 in Zmapqtl) and Composite Interval Mapping (CIM; Model 6 in Zmapqtl). Markers to be used as cofactors for CIM were selected by Forward-Backward stepwise regression (FB method in SRmapqtl). Two different criteria were considered to accept the presence of a QTL. First, the genome-wide significance threshold (Van Ooijen 1999) was calculated to identify putative QTLs. The LOD threshold established using this method was 4. Second, the consistent detection of a QTL on the same map position, for different isolates or independent disease evaluations on leaves and stems, was also considered for QTL acceptance. Uncertainty of the map position for the QTLs was calculated using a 2-LOD-support interval (Van Ooijen 1992). The QTLs detected in this work have been named *Af* (from *Ascochyta fabae*) followed by a number. We started with *Af3* since Roman et al. (2003) have previously mapped two QTLs for *A. fabae* resistance called *Af1* and *Af2* in a different population.

Results and discussion

Ascochyta blight resistance to two *A. fabae* isolates (CO99-01 and LO98-01) was investigated and compared. A disease test on ten faba bean lines indicated that both isolates differed at the pathogenic level since the ANOVA of the resistance data (data not shown) revealed a significant isolate × genotype interaction for all assessments (IT, DSL and DSS). This fact suggests a physiological specialization of these isolates, the isolate from Logroño being more aggressive as demonstrated by the highest DSL and DSS values, both in the susceptible parent (Vf 136) and the susceptible check (VS 172) (Table 1). Both isolates caused the same IT in the susceptible parent and the susceptible check (displaying large coalescing lesions), that were, however, more frequent (higher DSL and DSS) when inoculated with LO98-01.

A high significant correlation was found between IT and DSL for both CO99-01 (r=0.779) and LO98-01 (r=0.685) isolates. Similarly, high significant correlation between DSL and DSS for the CO99-01 isolate was also observed (r=0.745), while for isolate LO98-01 this correlation was moderate (r=0.489). IT and DSS showed a moderate level of correlation for both isolates, with r=0.592 and r=0.424 for CO99-01 and LO98-01 respectively. Finally, correlations between both isolates were moderate for each of the scales used.

Linkage map

A total of 103 marker loci segregating in the F_2 population were mapped into 18 linkage groups (LG). Out of these, four were isozyme loci (*Est-1*, *Pgd-1*, *Prx-1* and *Sod-1*), two were seed protein genes (legumins B3 and B4), three were microsatellites (GA-4, GAII59 and JF1-AG3) and 94 were RAPD markers. Linkage groups ranged from 2 to 16 maker loci, covering 1,308 cM of the faba bean genome. Out of the 18 linkage groups, six were assigned to specific chromosomes by means of physically located markers (Fig. 1). Since Pozarkova et al. (2002) adscribed the markers GA-4 and JF1-AG3 to chromosome 1, LG 1 was unambiguously assigned to the metacentric chromosome of the species. Similarly, legumins B3 and B4 were physically located on chromosome 3 (Macas et al. 1993a, 1993b; Vaz Patto et al. 1999) allowing the assignation of LGs 2 and 11 to this chromosome. Chromosomal location of LG 4 was possible due to the presence of the isozyme *Prx-1,* previously assigned to chromosome 5 (Torres et al. 1998). Likewise, the same authors located *Est-1* on chromosome 1 and *Sod-1* to chromosome 6, allowing the assignment of LGs 10 and 16 as well.

QTLs for ascochyta blight resistance to isolate CO99-01

Linear regression, Simple Interval Mapping (SIM) and Composite Interval Mapping (CIM) methods were used to identify QTLs for ascochyta blight resistance to *A. fabae* isolate CO99-01. Since results obtained by the three

Table 1 Phenotypic data obtained after the disease evaluation in the parental lines (29H and Vf 136), the susceptible check (VS 172) and the F_{2-3} progeny, using three evaluation scales: infection type (IT), disease severity on leaves (DSL) and disease severity on stems (DSS). Two different isolates are used, CO99-01 and LO98-01

Plant Material Statistical data	$F_{2:3}$				Vf 136 Average	29H Average	VS 172 Average
	Average	Variance	Maximum	Minimum			
IT CO99-01	2.9	0.49	4.3	1.4	5	0	5
IT LO98-01	2.8	0.99	4.5	0.0	5	0	5
DSL CO99-01	2.1	1.32	7.4	0.4	4.8	0.2	12.0
DSL LO98-01	2.7	4.50	11.0	0.0	17.5	0.0	16.9
DSS CO99-01	1.9	1.49	7.9	0.1	4.9	0.2	14.0
DSS LO98-01	1.5	1.97	6.2	0.0	17.5	0.0	19.7

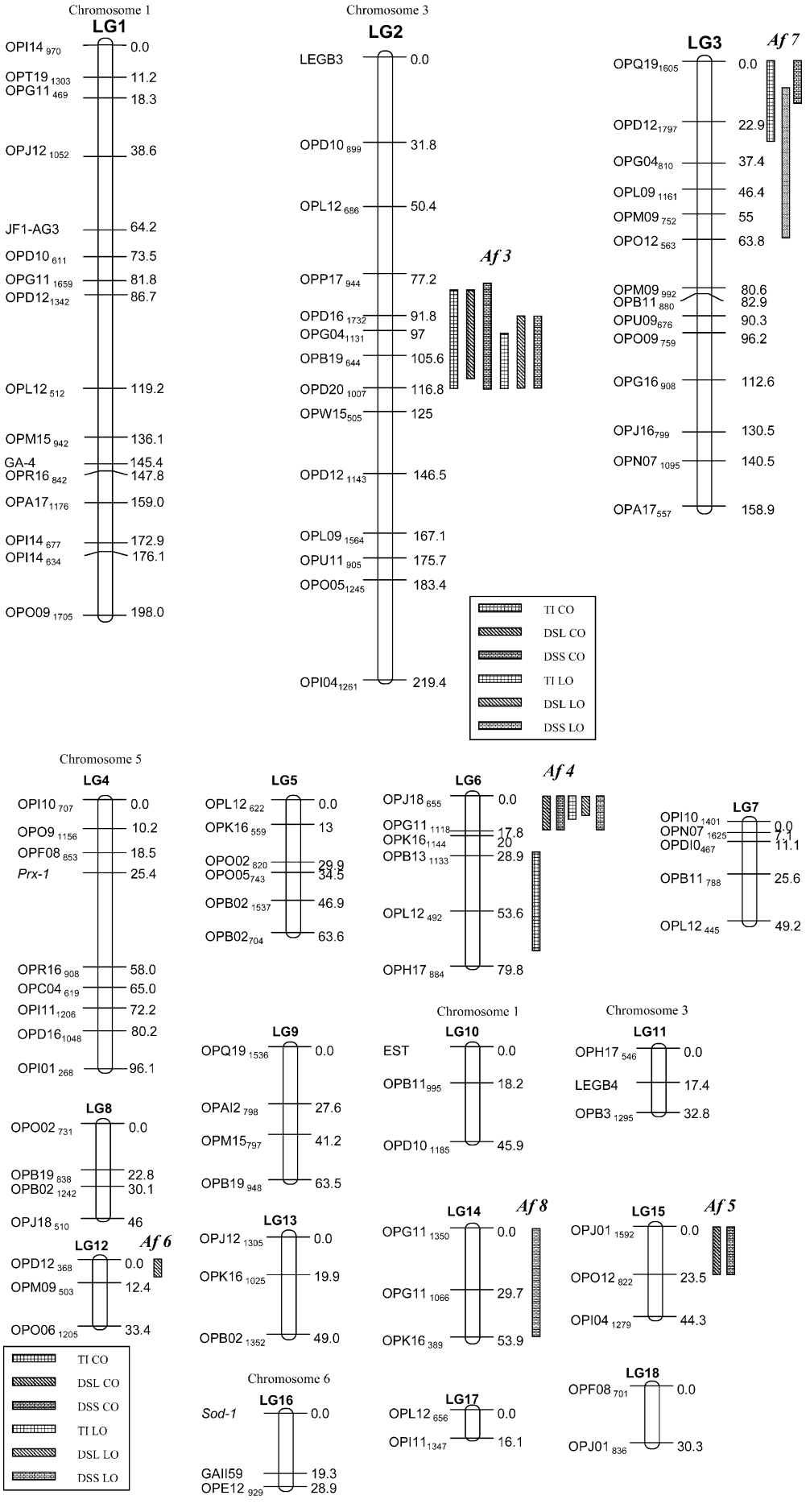

Fig. 1 Linkage map developed using the F_2 population 29H × Vf 136. Map positions are given in cM, using the mapping function of Kosambi (1944). *Bars* indicate the QTL locations for each assessment and isolate. *Length of bars* correspond to the 2 LOD support confidence interval based on the results of the CIM method, according to Van Ooijen (1992)

Table 2 Putative QTLs detected for ascochyta blight (*A. fabae* Speg.) resistance in faba bean (*V. faba* L.), using two different isolates (CO99-01 and LO98-01), by composite interval mapping (CIM)

QTL	Linkage group	Organ	Assessment	CO99-01			LO98-01		
				LOD[d]	%Exp[e]	Gene action[f]	LOD	%Exp	Gene action
Af3	LG2		IT[a]	4.8	13.4	PD	8.2	19.9	PD
		Leaves	DSL[b]	8.1	19.2	PD	9.0	22.4	PD
		Stem	DSS[c]	2.2	6.3	D	3.2	8.8	PD
Af4	LG6		IT	4.7	27.7	PD	5.1	11.7	PD
		Leaves	DSL	5.0	21.8	PD	4.5	28.9	PD
		Stem	DSS	2.9	27.2	PD	4.0	35.2	D
Af5	GL15		IT	–	–	–	–	–	–
		Leaves	DSL	3.0	30.1	OD	–	–	–
		Stem	DSS	3.6	36.1	D	–	–	–
Af6	GL12		IT	–	–	–	–	–	–
		Leaves	DSL	–	–	–	4.0	33.6	OD
		Stem	DSS	–	–	–	–	–	–
Af7	LG 3		IT	–	–	–	2.8	9.6	PD
		Leaves	DSL	–	–	–	2.2	6.2	PD
		Stem	DSS	–	–	–	7.2	44.4	D
Af8	LG 14		IT	–	–	–	–	–	–
		Leaves	DSL	–	–	–	–	–	–
		Stem	DSS	–	–	–	4.1	44.7	D

[a] Infection type
[b] Disease severity on leaves
[c] Disease severity on stems
[d] Peak value of the maximum LOD observed for the QTL
[e] Proportion of phenotype variance explained
[f] Gene action: D, dominance; PD, partial dominance and OD, over-dominance

methods were consistent, only the ones derived from the CIM method are presented (Table 2). Phenotypic values for IT, DSL and DSS revealed by the parents and their progeny are shown in Table 1.

Using the IT scale, two putative QTLs (*Af3* and *Af4*) in LGs 2 and 6 were detected (Table 2; Fig. 1). Their maximum LOD values were 4.8 and 4.7, respectively, and accounted for 13.4 and 27.7% of the phenotypic variation of the character. The same QTLs were consistently identified for DSL displaying LOD values of 8.1 and 5.0 (Table 2; Fig. 1). In this case, both QTLs explained 19.2 and 21.8% of the variation, respectively. Both regions might also be related with the stem resistance but LOD values were lower than 4, the significant threshold value chosen for declaring a QTL. A third region (*Af5*) could be related with resistance against this isolate in LG 15. Although LOD values obtained for DSS and DSL were lower than 4 as well (3.0 and 3.6, respectively), it seems that the co-appearance of the QTLs peak for both organs supports the existence of this QTL.

QTLs for ascochyta blight resistance to isolate LO98-01

Statistical methods to identify QTLs for resistance against isolate LO98-01 were the same as described above. Phenotypic data for disease evaluation using the three scales are presented in Table 1. Three putative QTLs were identified using DSL (*Af3*, *Af4* and *Af6*) on LGs 2, 6 and 12, respectively. LOD values for these QTLs were higher than 4 and explained 22.4, 28.9 and 33.6% of the phenotypic variation, respectively (Table 2). Out of these QTLs, *Af3* and *Af4* were additionally detected in a similar position using the IT scale in the evaluation of the resistance (Table 2; Fig. 1). In case of DSS, four regions located on LGs 2, 3, 6 and 14 might be associated with the resistance (*Af7*, *Af8*, *Af4* and *Af3*). *Af4, Af7* and *Af8* displayed a significant threshold value (LOD=4.0, 7.2 and 4.1) accounting respectively for 35.2, 44.4 and 44.7% of the variance of the trait (Table 2). Although *Af3* did not reach the LOD threshold for DSS (LOD=3.2), again the consistent localization in both leaves and stems may indicate that *Af3* is possibly acting in both organs.

Isolate-specific and organ-specific QTLs for ascochyta blight resistance

Ascochyta blight resistance to isolates CO99-01 and LO98-01 was resolved into six QTLs. *Af3* and *Af4* were effective against both isolates, *Af5* was only effective against isolate CO99-01, and *Af6*, *Af7* and *Af8* were only effective against isolate LO98-01 (Fig. 1; Table 2). Additionally, these QTLs differed in their action on leaves and stems. *Af3*, *Af4*, *Af5* and *Af7* were effective in both, leaves and stems, whereas *Af6* only was effective in leaves and *Af8* was only identified using stem evaluation.

QTLs *Af3* and *Af4* were effective against both isolates and were consistently detected in both leaves and stems. Although their peaks of LOD were lower than the

threshold level in the stem evaluation (DSS), the consistent detection in both parts of the plant using different isolates might support the action of these QTLs in both organs. *Af3* was ascribed to chromosome 3 since the legumin B3, included in the array, was previously assigned to this chromosome (Vaz Patto et al. 1999). Roman et al. (2003), analyzing the $F_{2:3}$ progeny of the cross Vf 6 × Vf 136, described a QTL (*Af1*) associated with ascochyta blight resistance on leaves in the same chromosome. The homology of the two regions has not been established since common markers in the vicinity of both QTLs are still not available. For this reason, we are transforming markers tightly linked to both QTLs into more specific markers to establish the possible identity of these QTLs. If we succeed, this would be the first example of the stability of QTLs for ascochyta blight resistance across different genetic backgrounds.

Af4 was also detected against both isolates using the three assessments (IT, DSL and DSS). However, the confidence interval of this QTL using IT with the isolate CO99-01 did not agree with the findings of the other five analyses (Fig. 1). Therefore, the most suitable location of this QTL would be determined by the agreement between the majority of the analyses.

Isolate-specificity was clearly revealed by QTLs *Af5* and *Af6*. *Af5* was only effective against isolate CO99-01 and *Af6* was only able to control isolate LO98-01. In addition, *Af5* was consistently identified in both organs of the plant while *Af6* was only effective in the leaves. In both cases, the QTLs were not detected using the IT scale. In the present study, both IT and DSL scales have been used for the evaluation of ascochyta blight resistance in leaves. Although both scales showed a high correlation, our data indicate that DSL might be more efficient than IT. This is not surprising since DSL is a quantitative evaluation, which allows establishing more precisely the disease severity. By contrast IT is a qualitative scale and therefore its discrimination power is lower than that of DSL. *Af5* and *Af6* could not be detected using IT, while both were revealed by scoring DSL. Our results are in agreement with the findings of Sillero et al. (2001) that concluded that the IT scale would underestimate the level of resistance since they found genotypes with large lesions (high IT values) but at a rather low quantity, resulting in low DS.

Af7 and *Af8* were also isolate-specific. *Af7* was detected in leaf and stem evaluations while *Af8* was only evident in stems. Our results indicate that while some QTLs are effective against more than one isolate (*Af3* and *Af4*) others seem to be isolate-specific (*Af5*, *Af6*, *Af7* and *Af8*). However, the question whether *Af3* and *Af4* are also isolate-specific will only be answered when a large number of isolates have been tested.

Similarly, resistance to ascochyta blight in leaves and stems appeared to be controlled by some common QTLs (*Af3*, *Af4*, *Af5* and *Af7*), while others were effective in only one organ (*Af6* and *Af8*). Other authors have also suggested the existence of different genes/QTLs controlling the resistance in each plant organ (Rashid et al. 1991b; Kharrat et al. 1997; Kohpina et al. 2000). All these studies evidenced the lack of correlation between leaf and stem assessments that might be caused by the environmental conditions. Although we have used controlled environmental conditions to minimise the environmental effects, the plant architecture itself may be influencing the development of the disease. For instance, Kohpina et al. (2000) reported that the spore suspension is sometimes held in the axil of the petiole and stem, increasing the likelihood of stem lesions or that lesions are often developed on younger leaves which had been folded at the time of inoculation. Consequently, it might be possible that some QTLs detected in a single organ (such as *Af6* and *Af8*) may be acting in others, as well. Therefore, although our results suggest that the differences observed between these two plant organs have a genetic basis, it is possible that the differences observed have purely environmental causes.

Final remarks

We have developed a molecular genetic linkage map, which has proved to be a valuable tool for the location of QTLs for ascochyta blight resistance. Although in several occasions *A. fabae* resistance was suggested to be under monogenic control (Rashid et al. 1991a; Kohpina et al. 2000), our results indicate that it is more adequate to consider this resistance as a quantitative trait since several QTLs have been detected. Although polygenic resistance is expressed quantitatively and selection is based on relatively small differences between plants, it is widely considered as the most valuable genetic resource for the development of long-lasting and effective resistance against all races of the pathogen.

We have detected two QTLs acting against both isolates studied, that might also be effective against a wider isolate spectrum. The other QTLs identified in the study were only effective against one of the tested isolates. Our results confirm the need to consider different isolates in a breeding program, since the resistance genes present in the resistant lines might be only effective against some of them. Therefore, preliminary knowledge on the existence of races and their virulence spectrum is needed.

Regarding the genetic control of resistance in leaves and stems, we have identified common QTLs in both organs, while others were only detected in one of them. This outcome suggests the existence of common and specific genetic systems to control ascochyta blight, although further studies are needed to provide insight into this question. Fine mapping of these resistance genes will help in marker-assisted selection for ascochyta blight resistance in *V. faba*, facilitating gene transfer and pyramiding of the genes into acceptable genetic backgrounds.

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