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Biochemical and genetic studies of two Heterodera avenae resistance genes transferred from *Aegilops ventricosa* to wheat

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Abstract Two *Heterodera avenae* resistance genes, *Cre2* from *Aegilops ventricosa* AP-1 and *Cre5* from *Ae. ventricosa* #10, were shown to confer a high level of resistance to the Spanish pathotype Ha71. No susceptible plants were found in the F_2 progeny from the cross between the two accessions of *Ae. ventricosa*, suggesting that their respective resistance factors were allelic. However, genes *Cre2* and *Cre5* apparently were transferred to a different chromosomal location in the wheat line H-93-8 and in the $6M^v(6D)$ substitution, respectively, as proved by F_2 segregation of their cross progeny. The induction of several defence responses during early infection by the same *H. avenae* pathotype in resistant lines carrying *Cre2* or *Cre5* genes was studied. Isoelectrofocusing (IEF) isozyme analysis revealed that peroxidase, esterase and superoxide dismutase activity increased after nematode infection, in roots of resistant lines in comparison with their susceptible parents. Differential induced isoforms were also identified when IEF patterns of resistant lines were compared. A DNA marker, absent in *Cre5*-carrying genotypes, was found to be linked, thought not very tightly, to the *Cre2* gene in the H-93-8 line. The differences observed between the *Cre2* and *Cre5* genes with respect to their chromosomal location in wheat introgression lines, de-toxificant enzyme induction and behaviour against different pathotypes, suggest they are different *H. avenae* resistance sources for wheat breeding.

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Introduction

The "cereal cyst nematode" (CCN), *Heterodera avenae* Wollenweber, is a pest that seriously affects cereal crops in many of the world's wheat-growing areas. Current methods of nematode control include nematicide treatments, crop rotation and other cultural practices. Soil fumigation can be effective, but is not profitable and causes environmental pollution. Integrated management of resistant-cultivars combined with crop rotation offers the cheapest way of effectively controlling this nematode (McIntosh 1998).

Interactions between pathogens and host plants are defined as compatible when disease symptoms result from the ability of pathogens to overcome host-defence responses. On the other hand, incompatible ones, resulting in the absence of symptoms, are determined by the interaction between a host resistance gene and a pathogen avirulence gene (Flor 1971). To-date, seven resistance genes to CCN, designated *Cre1* to *Cre7*, have been described in wheat (McIntosh et al. 2001). The *Cre1* gene has been characterised in the wheat line Aus10894/Loros (Slootmaker et al. 1974), while the *Cre2* to *Cre7* genes have been transferred to bread wheat from alien species. The genes *Cre2*, *Cre5* and *Cre6* were transferred from *Aegilops ventricosa* (Delibes et al. 1993; Jahier et al. 1996; Ogbonnaya et al. 2001), *Cre3* and *Cre4* from *Aegilops squarrosa* (Eastwood et al. 1991) and *Cre7* from *Aegilops triuncialis* (Romero et al. 1998). The designations of *Cre5* and *Cre7* have recently been accepted for the *CreX* and *CreAet* genes, respectively (McIntosh et al. 2001). Some of these genes confer only partial resistance to different CCN-pathotypes, and even those conferring full resistance may be overcome by newly-emerging pathotypes. The identification within alien germplasm of additional sources of resistance that could be employed in wheat-breeding programs is therefore an important goal.

Wild grass, *Ae. ventricosa* (syn. *Triticum ventricosum*) has long been used as a source of disease-resistance genes (RG) for bread wheat (*Triticum aestivum*). This species is an allotetraploid (2n = 4*x* = 28) with the genomic constitution $D^vD^vM^vM^v$, which is partially homologous to that of *T. aestivum* (AABBDD). Genes from the D^v genome are more easily transferred by recombination with the D genome, than those from the homoeologous but less-closely related M^v genome (Doussinault et al. 1983; Mena et al. 1993). So far, three dominant resistance genes, *Cre2, Cre5* and *Cre6*, have been transferred to wheat germplasm from two different accessions of *Ae. ventricosa*. The *Cre5* gene from *Ae. ventricosa* #10, carried on a $2M^v$ segment translocated on chromosome $6M^v$, was transferred to the wheat/*Ae. ventricosa* $6M^v(6D)$ substitution and $6M^v$ addition lines (Jahier et al. 1996, 2001). This gene confers partial resistance to French (Ha12 and Ha41) and Australian (Ha13) CCN-pathotypes (Dosba et al. 1978; Rivoal et al. 1986, 1993; Jahier et al. 2001; Ogbonnaya et al. 2001). RG analogs associated with *Cre5*, have also recently been isolated (Seah et al. 2000). The genes *Cre2* and *Cre6* were transferred from the M^v genome of *Ae. ventricosa* AP-1 to the H-93-8 line, a $5M^v(5A)$ and $7M^v(7D)$ substitution line, obtained from the cross [(*Triticum turgidum* × *Ae. ventricosa*) × *T. aestivum*] (Delibes et al. 1993; Mena et al. 1993; Ogbonnaya et al. 2001). The *Cre2* gene located on an unknown M^v chromosome, confers a high level of resistance to populations of *H. avenae* Ha71 (Spanish), Ha11 (British) and Fr1–Fr4 races (French), but was ineffective against HgI and HgIII (both Swedish) and the Australian Ha13 (Delibes et al. 1993; Ogbonnaya et al. 2001). On the other hand, the *Cre6* gene located on the $5M^v$ chromosome, which confers resistance to the Australian population Ha13, is ineffective against the Spanish pathotype Ha71 (Ogbonnaya et al. 2001).

Increased peroxidase, esterase and superoxide dismutase activity has been reported in various types of stress affecting plants, and it has been suggested as a defence mechanism against oxidative damage. In plants with resistance to nematodes, esterases, peroxidases and phenylalanine ammonia lyases increased after nematode infection, while a reduced level of superoxide dismutase occurred (Zacheo and Bleve-Zacheo 1995; Lambert et al. 1999; Andrés et al. 2001). Early histological and biochemical responses of roots from resistant line H-93-8 to the Spanish pathotype Ha71 have previously been reported, showing a strong correlation between cytological expression of resistance (hypersensitive response) and peroxidase and esterase activity (Andrés et al. 2001).

The aim of this paper is to compare resistance to CCN-pathotype Ha71 conferred by genes *Cre2* in H-93-8 and *Cre5* in $6M^v(6D)$, and to determine whether these genes are allelic in these introgression lines. We have studied the differential expression of some defence-response enzymes in infected/uninfected wheat roots, both in the presence and absence of these genes. We have also searched for DNA markers linked to *Cre2* to be used in marker-assisted pyramiding of different genes.

Materials and methods

Plant materials

Hexaploid H-93 lines derived from the cross [(*T. turgidum* ssp. *turgidum* cv Rubroatrum, H-1-1 × *Ae. ventricosa* AP-1) × *T. aestivum* cv Almatense, H-10-15] have previously been described (Delibes et al. 1993; Mena et al. 1993). The *T. aestivum* cv Moisson/*Ae. ventricosa* #10 addition lines in the cytoplasm of *Ae. ventricosa* #10 and Moisson, and the $6M^v(6D)$ substitution line, were provided by Dr. Dosba and Dr. Jahier (INRA, France). Hereafter the abbreviations *T. turgidum* H-1-1, H-10-15 and Moisson will be used.

The inheritance of the resistance derived from *Ae. ventricosa* was studied in F_2 plants from crosses between the resistant lines [$6M^v(6D)$ × H-93-8] and between their parents (*Ae. ventricosa* AP-1 × *Ae. ventricosa* #10). The cross (H-10-15 × H-93-8) was made in order to search in the F_2 generation for DNA markers linked with resistance conferred by the *Cre2* gene. Two generations per year were grown. All crosses were carried out in a greenhouse using standard manual procedures.

Nematode tests

Routine resistance screenings were carried out as described by Delibes et al. (1993) and Romero et al. (1998). The pathotype Ha71 of *H. avenae* was used in all the experiments described in this paper. Scores were expressed in terms of the number of females per gram of root. *T. aestivum* cultivar Anza was used as a susceptible control.

Tests for CCN resistance under field conditions were carried out at the Experimental Station "La Higueruela" (Toledo, Spain). The plot was naturally infested and the surface soil was homogenised before sowing. Individual plants from the F_1 and F_2 generations of all crosses and their parents were tested for resistance. Ratios of resistant to susceptible plants were compared using the chi-square test in order to determine the goodness of fit to the hypothesised ratios.

Resistance screening of the H-93-8 line, the disomic *T. aestivum*/*Ae. ventricosa* addition lines, the $6M^v(6D)$ substitution line and their parents was carried out by sowing individual plants into buried plastic cylinders (6-cm diameter, 20-cm high) that had been filled with sterilised soil and inoculated with 25 cysts collected at the same Experimental Station.

For isozyme studies, seedlings of resistant lines [H-93-8, $6M^v(6D)$ and $6M^v$], wheat parents (H-10-15 and Moisson) and the cultivar Anza, were grown in 3-cm-diameter Petri dishes containing 10 ml of sterilised sand and maintained at 17 °C in a growth chamber. Seedlings were subsequently divided into two groups. One was inoculated 48 h after germination, with approximately 100 juveniles/seedling of *H. avenae*, and the other was used as a control (uninoculated seedlings). Root sections taken 4, 7 and 14 days after infection, and an equal quantity of uninfected material, were cut out and used for isozyme analysis.

Enzyme analysis

Root tissue was ground up by a pestle in a small mortar with sand (1.5 mg of root per 1 μl of 20% sucrose solution) and extracted in an ultrasonic bath at 2°–4 °C for 10 min. Homogenates were centrifuged at 13,000 g for 10 min at 4 °C before application to the gel. Twenty five microliters of supernatant (about 15 μg/μl of protein) was immediately used for electrophoresis. Peroxidase (PER; EC 1.11.1.7), esterase (EST; EC 3.1.1.1) and superoxide dismutase (SOD; EC 1.15.1.1) isozyme systems were fractionated by isoelectric focusing (IEF). The electrophoresis was carried out at 4 °C using the Flat-bed apparatus FBE 3000 (Pharmacia). Polyacrylamide gels containing 10% glycerol and 2% w/v ampholytes Servalyt (Serva), were run at 1 W/cm length, with voltage limited to 3 kV. Samples were loaded upon the gel 1 cm

Table 1 Isoelectrofocusing conditions used for isozyme analysis

Isozyme system	Ampholyte pH range (ratio)	Gel measures		Electrode solutions	
		Length (mm)	Thickness (mm)	Cathode	Anode
PER	2–4/3–10/9–11 (1:5:1)	170	0.25	1 M NaOH	0.33 M Citric acid
	6–8/7–9/9–11 (1:1:1)	120	0.25	1 M NaOH	0.33 M Citric acid
	3–7	120	0.25	1 M NaOH	0.1 M Sulphuric acid
	2–4/3–5 (1:2)	170	0.25	0.1 M NaOH	0.04 M Glutamic acid
EST	3–7	120	0.25	1 M NaOH	0.33 M Citric acid
SOD	3–7	120	0.50	1 M NaOH	0.33 M Citric acid

from the anode. The electrophoretic conditions (gel measures, pH range of ampholytes and catholyte and anolyte solutions) used for the analysis of each isozyme system are shown in Table 1. The 120 mm-long gels were pre-focused at 0.5 kV/h and focusing was terminated at 4 kV/h. The 170 mm-long gels were pre-focused at 1 kV/h with focusing terminating at 12 kV/h. A minimum of four electrophoresis runs were performed, with extracts proceeding from different samples, in order to confirm the reproducibility of the isozyme patterns. The isoelectric points of the isozyme bands were determined before staining, by taking pH readings across the gel at 5-mm intervals using a surface electrode. Gels were fixed with 7% acetic acid solution. Peroxidase activity was visualised using the method described by Liu et al. (1990), esterase according to Liu and Gale (1994) and superoxide dismutase following Neuman and Hart (1986), except that pH 7.4 was used for the staining solution.

DNA extraction, amplification and cloning

Total genomic DNA was extracted according to the CTAB procedure described by Taylor and Powell (1982). Amplification with primers s2 (GGIGGIGTIGGIAAIAC IAC) and as3 (IAGIG-CIAGIGGIAGICC) was as described by Leister et al. (1996) and the products were cloned in the pGEM-T Easy vector (Promega) according to the manufacturer's indications. Amplification with primers 20 and 10 nucleotides long was carried out in a Perkin-Elmer 9700 Thermocycler with 0.5 U of Ampli*Taq* Gold (PE-Biosystem), 150 ng of DNA and 1 μM of each primer in a 20-μl volume, with 7 min initial denaturation at 94 °C. This was followed by five cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 2 min and a further 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min. The final step was at 72 °C for 7 min. PCR products were separated on a 2% agarose gel using a 1× TAE buffer and then stained with ethidium bromide.

Results

CCN- pathotype Ha71 reproduction on *Ae. ventricosa* and its derivatives in wheat

Tests carried out in plastic cylinders infested with 25 cysts of pathotype Ha71 of *H. avenae* showed total or nearly complete inhibition of nematode reproduction in both *Ae. ventricosa* accessions (#10 and AP-1) (Fig. 1). A high level of resistance to the same population was also observed in H-93-8, and in 6M^v addition and substitution lines in the cytoplasm of *Ae. ventricosa* #10 (Fig. 1) and of *T. aestivum* cv Moisson (data not shown). The degree of infection of Moisson and H-10-15, the parents of the resistant lines, was lower than in Anza, which showed more than 30 females per gram of root.

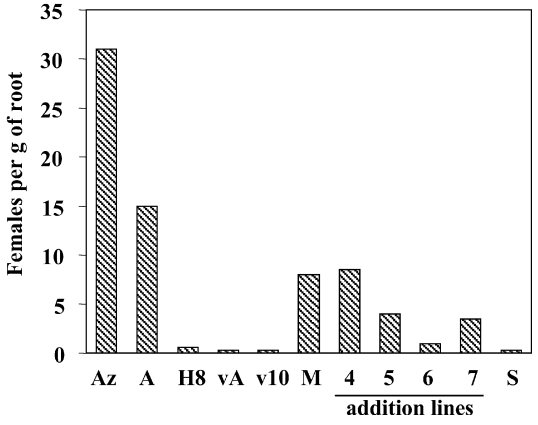

Fig. 1 Evaluation of the response to the Spanish pathotype Ha71 of *H. avenae* under controlled conditions of the stocks: *T. aestivum* cvs Anza (Az), H-10-15 (A) and Moisson (M); *Ae. ventricosa* AP-1 (vA) and #10 (v10); H-93-8 line (H8); addition lines *T. aestivum* cv Moisson/*Ae. ventricosa* #10: 4M^v (4), 5M^v (5), 6M^v (6) and 7M^v (7); substitution line 6M^v(6D) (S). Each bar represents the average of 4 to 14 plants per stock

Under natural infestation, no differences were observed between the level of resistance of *Ae. ventricosa* AP-1 and #10 accessions, on which again an almost complete failure of nematode reproduction was detected (Fig. 2A).

Inheritance of resistance derived from *Ae. ventricosa*

All F_1 and F_2 plants (286) from the cross (*Ae. ventricosa* AP-1 × *Ae. ventricosa* #10) were resistant (Fig. 2A). To determine whether the genes *Cre2* in H-93-8 and *Cre5* in the 6M^v(6D) substitution were allelic, these lines were crossed and the level of resistance of individual plants from the F_1 and F_2 generations and their parents was tested (Fig. 2B). The individual F_2 plants (131) were classified as either resistant or susceptible, using as a demarcation point the lower limit of the mean confidence interval (P = 95%) for the susceptible control *T. aestivum* cv Anza. A segregation between resistant and susceptible plants that fit 15:1 [$\chi^2_{df:1}$ =1.88 ($0.1 < P < 0.2$)] was obtained, indicating that *Cre2* and *Cre5* are not allelic.

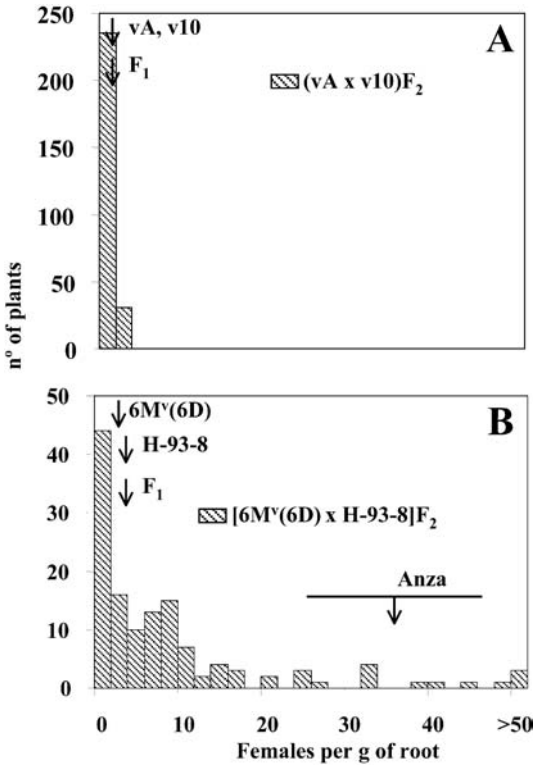

Fig. 2A, B Distribution of *H. avenae* (pathotype Ha71) infestation under natural conditions of F_2 individual plants of the crosses: **A** *Ae. ventricosa* AP-1 × *Ae. ventricosa* #10 (286 plants) and **B** $6M^v(6D)$ × H-93-8 (131 plants). In the upper part of each panel the average (*vertical arrow*) is shown for *Ae. ventricosa* #10 (v10, 10 plants); *Ae. ventricosa* AP-1 (vA, 10 plants); H-93-8 (10 plants); $6M^v(6D)$ substitution line (6 plants) and 6 and 4 plants from F_1 generations of the crosses (*Ae. ventricosa* AP-1 × *Ae. ventricosa* #10) and [$6M^v(6D)$ × H-93-8], respectively. *T. aestivum* cv Anza was used as a susceptible control (10 plants)

DNA markers

DNA from *Ae. ventricosa* AP-1 was amplified by PCR using two specific primers (s2 and as3) for the Resistance Gene Analogs (RGA) gene family. From all the clones obtained, seven fragments (Rae1-7, accession numbers EMBL AJ249943 to AJ249949) showed similarities with the fragment from Kinase-1 to GLPLAL motifs into the nucleotide binding site (NBS) of disease resistance genes, but none was specific for the *Cre2*-carrying genotypes (*Ae. ventricosa* AP-1 and H-93-8). Primers (20 nucleotides long) were designed from the 3′ terminal sequence of these fragments and used together with random primers (10 nucleotides long), to amplify the downstream NBS. From 700 primer combinations, one derived Rae2 (RAE2c, TAAAGCACTAAAAGGCCTCC and ILB470, AGGAGCTGGG) produced a 644-bp band (accession number EMBL AJ430696) that was present in *Ae. ventricosa* AP-1 and in the H-93-8 line; but was absent in the amplified products of the susceptible wheat parents (*T. turgidum* H-1-1 and *T. aestivum* H-10-15), in some susceptible H-93 lines (10, 22, 33 and 35) and in the $6M^v$ addition line, carrying the *Cre5* gene (Fig. 3). The same

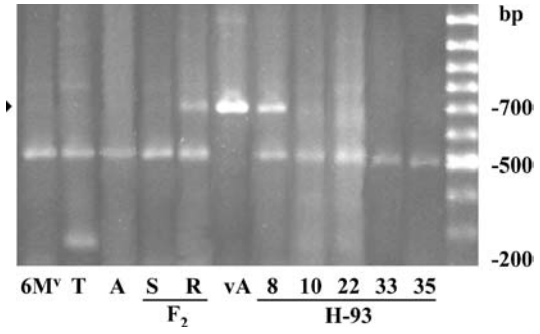

Fig. 3 Amplification products obtained using primers RAE2c and ILB470 on DNAs of the following lines: $6M^v$ addition line ($6M^v$); *T. turgidum* H-1-1 (T); *T. aestivum* H-10-15 (A); F_2 susceptible (S) and resistant (R) plants to *H. avenae* from the cross (*T. aestivum* H-10-15 × H-93-8); *Ae. ventricosa* AP-1 (vA) and H-93 lines –8 (8), –10 (10), –22 (22), –33 (33), –35 (35). The *Cre2* specific band is indicated by the *arrowhead*

primers were also used for amplifying DNA from 34 individual F_2 plants from the (H-10-15 × H-93-8) cross that had previously been classified as either resistant (26) or susceptible (8) in a field test. The marker was absent in all the susceptible, and present in 19 of the resistant, F_2 plants (Fig. 3).

Antioxidant isozymes

IEF isozyme patterns revealed an early response of defence enzymes (peroxidase, esterase and superoxide dismutase) in infected roots of H-93-8, $6M^v(6D)$ substitution and $6M^v$ addition resistant-lines (incompatible interactions). Changes in isozyme patterns were detected starting from 4-days after nematode infection, but they were more evident at 7 days.

The most significant alterations were observed in the peroxidase system, which was fractionated using four pH ranges (Table 1). New bands or increased activity of existing isoforms were detected (Fig. 4A and B). Figure 4A shows the peroxidase isozyme patterns (PER), fractionated using full-range ampholytes (pH 2–11), of roots 7-days after infection. A set of bands with isoelectric points (pIs) between 7.0 and 9.0, and with pIs 5.5 and 5.8, were induced in response to infection in incompatible interactions, i.e. H-93-8 and $6M^v(6D)$, though some of these also showed increases in compatible interactions (H-10-15 and Moisson). A better resolution of the basic peroxidases was obtained using a 6–11 pH range of ampholytes (Fig. 4B). Although the majority of the induced bands were common to H-93-8 and $6M^v(6D)$ resistant lines, only several increased in the latter. The $6M^v$ addition line showed the same induction PER-pattern as the $6M^v(6D)$ substitution (data not shown). No important changes were detected in IEF patterns obtained using a 2–5 and 3–7 pH-range of ampholytes as compared with the full range (data not shown). No significant differential PER expression was noted in response to

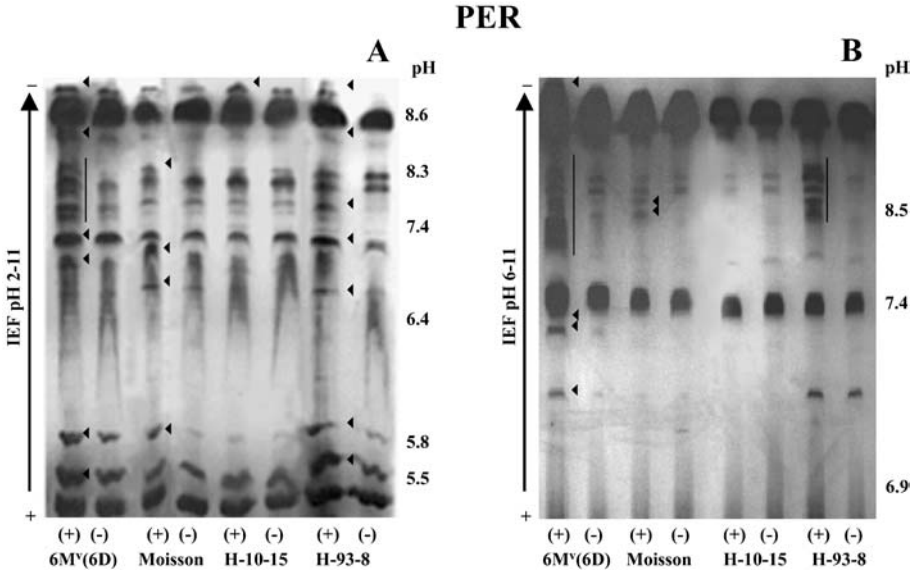

Fig. 4 Peroxidase isozyme IEF-patterns of roots taken 7 days after infection by *H. avenae* juveniles (+) and of uninfected roots as a control (–): **A** full pH range (2–11 ampholytes); **B** basic pH range (6–11 ampholytes). Stocks: H-93-8 and 6M^{v}(6D) resistant lines, and their susceptible parents, *T. aestivum* cvs H-10-15 and Moisson. New bands or those of increased intensity from infected roots are indicated by *arrowheads or vertical lines*

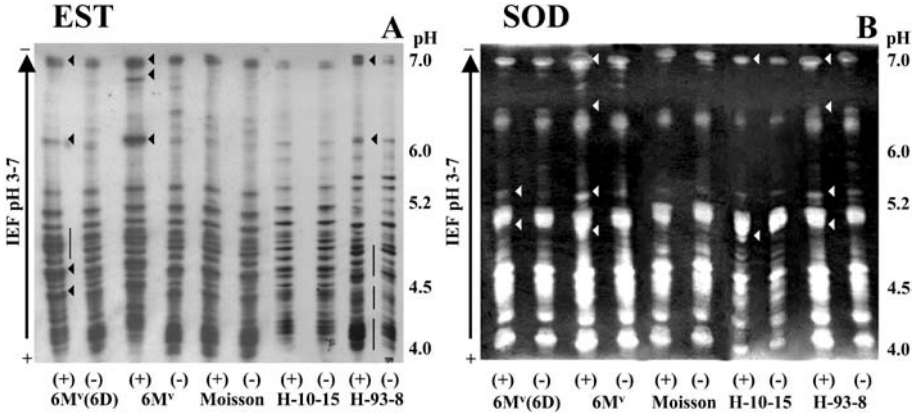

Fig. 5 Esterase (**A**) and Superoxide dismutase (**B**) isozyme IEF-patterns, of roots taken 7 days after infection by *H. avenae* juveniles (+) and of uninfected roots as a control (–), of the stocks: H-93-8, 6M^{v}(6D) substitution and 6M^{v} addition resistant lines, and their susceptible parents *T. aestivum* cvs H-10-15 and Moisson. Bands with increased intensity from infected roots are indicated by *arrowheads or vertical lines*

infection at 4, 7 and 14 days in the compatible interaction Anza-Ha71 using the four pH gradient gels. A slight increase in peroxidase activity was detected in interactions with Moisson and H-10-15, though this disappeared at 14 days. However, the early changes detected in the incompatible interactions remained with the same intensity at 14 days (data not shown).

Esterase (EST) and superoxide dismutase (SOD) isozyme systems from roots at 4, 7 and 14 days after nematode infection were fractionated by IEF in the pH 3–7-range gels. Isozyme patterns were similar from 4 to 14 days. The results shown are those at 7 days after infection (Fig. 5A and B). Increases in activity (indicated by arrowheads) in response to infection were observed in both isozyme systems in the resistant genotypes, i.e. H-93-8, 6M^{v}(6D) substitution and 6M^{v} addition lines. However, no significant changes were detected in Anza, Moisson and H-10-15 wheat roots when they were compared with their uninfected controls. The esterase bands focused around pH 6.0–7.0 and pH 4.0–5.0 and were more intense in all infected resistant lines than in uninfected controls, and differences in some band activity was also detected among resistant lines (Fig. 5A). The same SOD isozyme bands with pIs close to 5.0, 5.2, 6.5 and 7.0, increased in activity in the roots of the H-93-8 line and in the 6M^{v} addition, whereas in the 6M^{v}(6D) substitution only the two most-acidic were induced (Fig. 5B).

Discussion

The high level of resistance to the Spanish pathotype Ha71 reported for accessions of *Ae. ventricosa* #10 and AP-1 was comparable with that described by Ogbonnaya

et al. (2001) for the same two accessions against the Australian pathotype Ha13.

The high levels of resistance conferred by the genes *Cre2* in H-93-8 and *Cre5* in $6M^v$(6D) substitution and $6M^v$ addition lines were very similar under controlled conditions and natural infestation. Even so, the attack was generally stronger under the latter conditions (Figs. 1 and 2B). The effectiveness of *Cre2* in line H-93-8 against the pathotype Ha71 was confirmed (Delibes et al. 1993). Moreover, this is the first time that the high level of resistance conferred by the *Cre5* gene is described. Previous studies have demonstrated differences between *Cre2* and *Cre5* with respect to their behaviour against other CCN-pathotypes. *Cre2* is more effective than *Cre5* against the French pathotype Ha41 as described by Jahier et al. (1996). An intermediate level of resistance to the Australian pathotype Ha13 in *Cre5*-carrying genotypes, including the $6M^v$(6D) substitution and the $6M^v$ addition lines, was also reported, whereas under the same conditions *Cre2* did not confer resistance (Ogbonnaya et al. 2001). In this study the $6M^v$ addition line showed a lower level of resistance than the $6M^v$(6D) substitution, but this value was higher than those found in other available disomic *T. aestivum*/*Ae. ventricosa* addition lines carrying chromosomes $4M^v$, $5M^v$ and $7M^v$ (Fig. 1). This result agrees with a number of studies in *Triticum*, which revealed that the expression of resistance genes against pathogens is reduced when they are transferred from a lower to a higher ploidy level (Hanušová et al. 1996). The partial resistance found in H-10-15 and Moisson (parents of resistant lines) confirms in the first case (Delibes et al. 1993; Romero et al. 1998), and suggests, in the second, the presence of factors that affect resistance expression.

We found no susceptible plants among the 286 screened for resistance in the F_2 generation from the cross (*Ae. ventricosa* AP-1 × *Ae. ventricosa* #10). If only one gene in each accession confers resistance to the pathotype Ha71, this result would be consistent with the hypothesis that *Cre5* and *Cre2* are allelic genes in *Ae. ventricosa* #10 and AP-1, respectively. However, we cannot rule out the possibility of other loci affecting resistance to the same pathotype. The existence of another resistance gene (*Cre6*) in *Ae. ventricosa* AP-1 and the H-93-8 line was previously demonstrated, but this was ineffective against the pathotype Ha71 (Ogbonnaya et al. 2001). In the segregation of the resistance trait in the F_2 progeny from the cross [$6M^v$(6D) × H-93-8], which fitted a 15R:1S ratio, we found some very susceptible F_2 plants. The hypothesis that *Cre2* and*Cre5* genes are allelic in H-93-8 and $6M^v$(6D) lines must therefore be rejected. A high proportion of F_2 plants from this cross had null-infection, while others showed few females, suggesting the existence of some resistant plants carrying both genes (*Cre2* and *Cre5*) and others with only one. The slightly higher number of susceptible plants in this F_2 population with respect to the expected segregation of 15R:1S, could be explained in terms of the known irregular meiosis of the H-93-8 line and its hybrids (Mena et al. 1993). This deviation may have resulted from a poor transmission of the wheat chromosome carrying the alien segment containing the *Cre2* gene in the H-93-8 line, as previously described for the *Cre6* gene (carried by chromosome $5M^v$) in the same introgression line (Ogbonnaya et al. 2001).

Molecular markers can support classical breeding of crop plants by shortening selection times (Mohan et al. 1997). The resistance 26R:8S segregation in F_2 progeny from the cross (H-10-15 × H-93-8) confirms the hypothesis of a quasi-dominant gene in line H-93-8 (Delibes et al. 1993). The F_2 distribution of the PCR marker and CCN-resistance in the same cross was not that expected for two independent loci. This result could be compatible with the linkage between the marker and the *Cre2* resistance gene in line H-93-8. Evidence of linkage was found in all clearly susceptible plants (with more than 15 females per plant), whose DNA profiles never showed the PCR marker. However, seven plants classified as resistant did not carry the marker, indicating that linkage between the two traits was not very tight. This result would suggest recombination between the introgressed chromosome of H-93-8, carrying both DNA segments, and a wheat chromosome, as previously described by Mena et al. (1993) for other H-93 lines (1, 3, 18, 33 and 51). The DNA marker showed sequence similarity with a BAC-clone fragment from *Triticum monococcum* (accession number AF459639.1) containing no described resistance gene.

Several pieces of evidence indicate that *Cre2* and *Cre5* map to different chromosome locations in H-93-8 and $6M^v$(6D) lines. Resistance conferred by *Cre2* and *Cre5* genes segregated independently in the F_2 progeny from the cross [$6M^v$(6D) × H-93-8], and the DNA marker linked to *Cre2* was absent in *Cre5*-carrying genotypes. The *Cre5* gene is located on a $2M^v$ segment translocated into chromosome $6M^v$, while no RFLP or isozyme markers for chromosomes $2M^v$ and $6M^v$ were detected in the line H-93-8 (Mena et al. 1993). On the other hand, the DNA marker linked to *Cre2* was absent in H-93 lines carrying $4M^v$ (H-93-33), $5M^v$ (H-93-35) and $7M^v$ (H-93-10 and H-93-22) chromosomes (Fig. 3). The *Cre2* gene was previously associated with the M^v genome but was not assigned to a particular chromosome (Delibes et al. 1993). From the present data its chromosomal location remains unknown.

The results reported in this paper clearly confirm previous observations on resistance conferred by the *Cre2* gene. The *H. avenae* pathotype Ha71 was unable to overcome its resistance mechanism in line H-93-8; and PER, EST and SOD activity increased in roots from this line after nematode infection (Delibes et al. 1993; Andrés et al. 2001). No studies of this type had previously been carried out on the above mentioned *Cre5* gene. Following nematode infection, changes in the same isozyme systems occurred in resistant and in susceptible plants, but they were greater and generally more precociously expressed in the former. Early induction of enzymes in response to infection in lines carrying *Cre2* or *Cre5* was very similar, though certain isoforms were differentially expressed. A

large increase in cathodic and anionic peroxidases, the latter associated with the lignification process, was found in early nematode infection as previously described by other authors (Melillo et al. 1992; Zacheo et al. 1993; Andrés et al. 2001). This response was more-intense in incompatible than in compatible interactions, and new isoforms or increases in specific peroxidase bands were seen. Some of these changes were different when we compared resistant lines. Similarly, the intensity of certain esterase bands increased in different resistant lines after infection, though no new isoform was detected. The small alterations in SOD-IEF profiles induced in response to infection were very similar in all resistant lines.

The cultivar Anza, which had previously demonstrated a fully compatible interaction against the Ha71 pathotype (Bleve-Zacheo et al. 1995; Romero et al. 1998; Andrés et al. 2001), showed no significant differences between infected and non-infected plants at 4, 7 and 14 days after infection, in all the enzyme systems studied. The partial resistance of H-10-15 and Moisson could explain minor changes in IEF enzyme profiles found in some isozyme systems between infected and uninfected plants. Our conclusion is similar to those of Lambert et al. (1999) with respect to the *Mi* gene in tomato; the products of some resistance genes with increased expression (isozyme activity) early after CCN infection could be involved in nematode resistance mediated by RG, and others could form part of a more-general defence system that is present in both resistant and susceptible wheat.

Altogether, the differences reported between *Cre2* and *Cre5* in the resistant lines H-93-8 and $6M^{v}(6D)$, with respect to their chromosomal location, de-toxificant enzyme induction and their previously described behaviour against various pathotypes, suggest that the two genes are different sources of resistance for wheat breeding. This finding is significant because new sources of resistance provide breeders with alternative genes against newly emerging pathotypes. Both genes could be introduced in the same wheat cultivar (gene pyramiding) before they are deployed. Sequential or simultaneous release of cultivars, carrying each individual gene, could also be effective.

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