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Review. Characterization and selection of hexaploid wheats containing resistance to *Heterodera avenae* or *Mayetiola destructor* introgressed from *Aegilops*

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Abstract

Cereal cyst nematode (CCN, *Heterodera avenae*) and Hessian fly (HF, *Mayetiola destructor*) are two major pests affecting wheat crops worldwide including important cereal areas of Spain. *Aegilops ventricosa* and *Ae. triuncialis* were used as donors in a strategy to introduce resistance genes (RG) for these pests in hexaploid wheat (*Triticum aestivum* L.). Two 42 chromosomes introgression lines have been derived from *Ae. ventricosa*: H-93-8 and H-93-33 carrying genes *Cre*2 and *H*27 conferring resistance to CCN and HF, respectively. Line TR-3531 with 42 chromosomes has been derived from *Ae. triuncialis* and carries RGs conferring resistance for CCN (*Cre*7) and for HF (*H*30). Alien material has been incorporated in lines H-93 by chromosomal substitution and recombination, while in line TR-3531 homoeologous recombination affecting small DNA fragments has played a major role. It has been demonstrated that *Cre*2, *Cre*7, *H*27 and *H*30 are major single dominant genes and not allelic of other previously described RGs. Biochemical and molecular-biology studies of the defense mechanism triggered by *Cre*2 and *Cre*7 have revealed specific induction of peroxidase and other antioxidant enzymes. In parallel to these basic studies advanced lines carrying resistance genes for CNN and/or HF have been developed. Selection was done using molecular markers for eventually «pyramiding» resistance genes. Several isozyme and RAPD markers have been described and, currently, new markers based on transposable elements and NBS-LRR sequences are being developed. At present, two advanced lines have already been included at the Spanish Catalogue of Commercial Plant Varieties.

Additional key words: *Aegilops triuncialis*, *Aegilops ventricosa*, cereal cyst nematode, Hessian fly, resistance genes, *Triticum aestivum.*

Resumen

Revisión. Caracterización y selección de trigos hexaploides con resistencia a *Heterodera avenae* o *Mayetiola destructor* transferida desde *Aegilops*

El nematodo del quiste de los cereales (CCN, *Heterodera avenae*) y el mosquito del trigo (HF, *Mayetiola destructor*) son dos plagas que afectan a los cultivos de trigo incluyendo importantes áreas cerealistas en España. *Aegilops ventricosa* y *Ae. triuncialis* se han usado como donantes en una estrategia para introducir genes de resistencia (RG) para estas plagas en trigo hexaploide (*Triticum aestivum* L.). Por una parte, a partir de *Ae. ventricosa* se han generado dos líneas de introgresión de 42 cromosomas: H-93-8 y H-93-33, con los genes *Cre*2 y *H*27 que confieren resistencia a CCN y HF, respectivamente. Por otra, se ha generado la línea TR-3531 de 42 cromosomas, derivada de *Ae. triuncialis*, con RGs para CCN (*Cre*7) y HF (*H*30). La introgresión en las líneas H-93 se ha producido por sustitución cromosómica y recombinación, mientras que en la línea TR-3531 ha sido fundamentalmente por la recombinación homeóloga de pequeños fragmentos de ADN. *Cre*2, *Cre*7, *H*27 y *H*30 son genes dominantes simples, no alélicos a otros RGs previamente descritos. Estudios bioquímicos y de biología molecular sobre las defensas inducidas por *Cre*2 y *Cre*7 han revelado que las peroxidasas y otras enzimas antioxidantes se inducen de forma específica. En paralelo a

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estos estudios básicos, se han desarrollado líneas avanzadas con resistencia para CNN y/o HF. La selección fue realizada usando marcadores moleculares que también han sido utilizados para la «piramidación» de RGs. Hasta el momento se han descrito varios marcadores isoenzimáticos y RAPD y actualmente se están desarrollando nuevos marcadores basados en transposones y secuencias NBS-LRR. Se han incluido dos líneas avanzadas en el Catálogo de Variedades Comerciales.

Palabras clave adicionales: *Aegilops triuncialis, Aegilops ventricosa,* genes de resistencia, mosquito del trigo, nematodo de quiste de los cereales, *Triticum aestivum.*

Introduction[1]

The transfer of genetic material from wild to cultivated plants has been extensively exploited to introduce resistance to different pests and diseases and other desirable agronomic traits into productive cultivars. Transfer of alien genes to hexaploid bread wheat (*Triticum aestivum* L., 2n = 6x = 42) through hybridization with various grass genera has significantly contributed to its genetic improvement. The wild grass genus *Aegilops* has been a particularly valuable source of disease and pest resistance genes (Gill *et al*., 1985). Crop rotations and application of pesticides can reduce the pest populations; however under economic and environmental constraints the best strategy is to use resistant cultivars.

Cereal cyst nematode (CCN, *Heterodera avenae*, Woll.) is a serious cereal root pathogen that affects many agricultural areas all over the world, and is particularly widespread in soils of Western Europe. Most CCN-resistance genes (*Cre*) have been identified in wheat related species within the tribe *Triticeae*, and only two of them (*Cre*1 and *Cre*8) have been found in hexaploid wheat (McIntosh *et al*., 2003). *Cre* genes from genus *Aegilops* have been transferred to wheat: *Cre*2, *Cre*5 and *Cre*6 from *Ae. ventricosa* (Delibes *et al*., 1993; Jahier *et al*., 1996; Ogbonnaya *et al*., 2001), *Cre*3 and *Cre*4 from *Aegilops tauschii* (Eastwood *et al*., 1991, 1994) and *Cre*7 from *Aegilops triuncialis* (Romero *et al*., 1998).

Hessian fly (HF, *Mayetiola destructor*, Say) is one of the most destructive pests of wheat crops. First described in the 19th century in Spain (Herreros, 1896), it causes extensive damage to wheat in main cereal growing areas in the Iberian peninsula and North of Africa (Castañera, 1985). In the South-West of Spain, where the main crop is winter cereal (approximately 150,000 ha), *M. destructor* attacks almost exclusively the wheat (Del Moral *et al*., 2002), two generations per year occur in infested fields (Delibes *et al.*, 1997). A gene-for-gene resistance mechanism involving mostly dominant alleles (*H* genes) has been demonstrated (Hatchett and Gallun, 1970). To date, 33 different HF resistance genes have been identified (*H*1-*H*32 and *H*dic) (McIntosh *et al*., 2003; Liu *et al*., 2005). Insect biotypes occur in nature as a result of selection by exposure to resistant cultivars.

The primary objective of our group is to obtain wheat genotypes resistant to the cereal cyst nematode and to the Hessian fly introgressing resistance genes (RGs) from *Aegilops* species and developing molecular markers to aid the selection process. Because *H. avenae* and *M. destructor* depend on wheat for their survival, resistant cultivars generate strong selection pressure on them. This results in the evolution of new avirulence alleles forcing wheat breeders to find new resistance sources. Moreover, the expression of enzymes associate to the subsequent defensive responses is being characterized.

Wheat/*Aegilops* introgression lines

Different studies from our laboratory have shown the effectiveness of a method to transfer genes from the wild grass *Ae. ventricosa* to the cultivated wheat *T. aestivum* (Doussinault *et al*., 1983; Mena *et al*., 1993). This method was developed by Manuel Alonso Peña in the sixties and involves crossing the donor, *Ae. ventricosa* (genomes $D^vD^vN^vN^v$) with *T. turgidum* (AABB), which act as a bridge, followed by the rescue of the sterile ABD^vN^v hybrid with pollen from the recipient species *T. aestivum* (AABBDD). Plants from

[1] Abbreviations used: AOS (active oxygen species), BOE (Boletín Oficial del Estado), CCN (cereal cyst nematode), *Cre* (cereal root eelworm), EST (esterase), HF (hessian fly), HR (hypersensitive response), IEF (isoelectrofocusing), MAS (markers assisted selection), OEVV (Oficina Española de Variedades Vegetales), PER (peroxidase), RG (resistance genes), SOD (superoxide dismutase), SW (south-west), UHFN (Uniform Hessian Fly Nursery).

this cross generated fertile and stable lines with 42 chromosomes after subsequent repeated selfing.

Different studies have shown that genetic material from the donor species has been incorporated into the transfer lines (H-93) both by chromosomal substitution and recombination (Mena *et al*., 1993). Using this approach, resistance genes to the fungi *Pseudocercosporella herpotrichoides* and *Blumeria graminis*, to *M. destructor*, and to *H. avenae*, have been introduced into wheat chromosomes (Doussinault *et al*., 1983; Delibes *et al*., 1987, 1993, 1997; Mena *et al*., 1992). The substitution line H-93-8 (5A/5N^{v} and 7D/7N^{v}) shows a high resistance level against *H. avenae* Spanish pathotype Ha*71*, four French races and one British pathotype, but is susceptible to two Swedish and one Australian pathotypes. Resistance to Ha71 is inherited as determined by a single dominant factor (*Cre2*) originally located in the N^{v} genome (Delibes *et al*., 1993; Andrés *et al*., 2001). The *Cre*6 gene, characterized in collaboration with an Australian group, is located on 5N^{v} chromosome of lines H-93-8 and H-93-35 (substitution 5D/5N^{v}), and confers a high resistance level to the Australian pathotype but it is ineffective against the Spanish Ha*71* (Ogbonnaya *et al*., 2001). Finally, the line H-93-33 (substitution 4D/4N^{v}) shows high level of resistance to *M. destructor* conferred by a single dominant gene (*H*27) in chromosome 4N^{v} (Delibes *et al.,* 1997).

In order to verify whether the method could be generalized to transfer genetic material among species with different ploidy level, it was used *Ae. triuncialis* (genomes CCUU) as a donor species and the same bridge and recipient species as before (Romero *et al*., 1998). In this case, enhanced homoeologous recombination should occur due to the known ability of the C genome to suppress the Ph diploidization mechanism of *T. aestivum* and *T. turgidum* (Kimber and Feldman, 1987) and the recently described gametocidal (Gc) action of the 3C chromosome that cause chromosome breakage in the gametes lacking them (Enzo, 2007). During the past 15 years Gc chromosomes have been utilized for the rearrangement of alien chromosomes added to common wheat. Plants with 28-41 chromosomes were obtained from this cross and by repeated selfing of one of those with 41 chromosomes, a line with 42 chromosomes was derived (TR-3531). This line has been characterized by isozyme analyses and cytomolecular methods. All evidences support that alien genetic material has been incorporated via homoeologous recombination affecting small DNA fragments (unpublished data). TR-3531 carries DNA fragments from chromosomes 4U, 5U and 7U, as well as high resistance level to *H. avenae* (pathotype Ha71) and to *M. destructor*. These resistances are determined by the single and dominant genes *Cre*7 and *H*30, respectively (Romero *et al*., 1998; Martín-Sánchez *et al*., 2003). *Cre*7 confers CCN resistance to three French, two Swedish and two Spanish pathotypes.

Antioxidant enzymes

Resistant plants respond to nematode infection by activating a number of inducible responses that are thought to be disease resistance related. Incompatible interaction between H-93-8 or TR-3531 lines and the CCN induces hypersensitive response (HR) with previous formation of syncitial cells and active oxygen species (AOS). Plants possess both enzymatic and non-enzymatic antioxidant defense systems to counteract AOS generated under stress conditions. The antioxidant enzymes include peroxidase (PER, EC 1.11.1.7), esterase (EST, EC 3.1.1.2) and superoxide dismutase (SOD, EC 1.15.1.1). Isoelectrofocusing (IEF) isoenzyme analysis, four and seven days after infection, revealed that PER, EST and SOD activities increased with the time in roots of lines H-93-8 and TR-3531, carrying *Cre*2, *Cre*5 and *Cre*7 genes, respectively, in comparison with the susceptible control (Andrés *et al*., 2001; Montes *et al*., 2003, 2004). Nematode infection preferentially enhances the activity of PER system and in advanced lines carrying *Cre*2 and *Cre*7, PER patterns are used to determine the presence of both RGs (see Fig. 1 for advanced lines ID-2150 carrying *Cre*2 and T-2003 carrying *Cre*7). The notable increase in cationic and anionic PER further support the participation of lignification in the non-host resistance. Peroxidase mRNA transcripts, on infected and uninfected H-93-8 and a susceptible control, have also been quantitatively analyzed by Northern using a peroxidase probe obtained by PCR. A significant increase of peroxidase mRNA level is observed seven days post-inoculation. Qualitative root specific expression pattern of peroxidase genes has been determined by cloning and sequencing RT-PCR products. All induced peroxidases show a C-terminal extension, which appears to mediate vacuolar import in plants (Welinder *et al*., 2002). The peroxidase activity patterns and transcript accumulation profiles analyzed suggest a role of peroxidase in resistance, probably in cell wall cross linking.

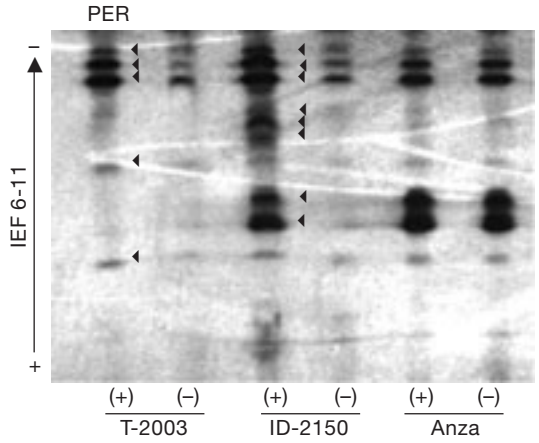

Figure 1. Peroxidase isozyme patterns obtained by isoelectrofocusing (IEF) using basic pH range of ampholytes of *T. aestivum* cv Anza (susceptible) and two advances lines carrying *Cre*2 (ID-2150) and *Cre*7 (T-2003) resistance genes at seven days infected (+) by *H. avenae* juveniles and uninfected roots (–) as control. Arrowheads indicate the new or enhanced bands in infected roots in comparison to the control.

Markers assisted selection (MAS)

When interspecific hybridization between the donor and recipient species is used as transfer strategy, the introgression of resistance genes (RG) from alien species into breeding material often dramatically reduced agronomic performance. To get rid of negative traits from the donor plant, the progeny is backcrossed several times with the elite breeding line. This is a time-consuming and not always successful strategy. Molecular markers linked to the RG can improve the selection efficiency through the backcrossing process and serve to pyramid major resistance genes. DNA and isozyme markers linked to genes *Cre*2, *Cre*6, *H*27 and *H*30 were developed for marker assisted selection (MAS). Two DNA markers were linked to *Cre*2 and *Cre*6 in H-93-8 and H-93-35 lines, respectively (Ogbonnaya *et al*., 2001; Montes *et al*., 2003) and two isozyme markers corresponding to homoeologous group 4 were linked to *H27* and *H30* in H-93-33 and TR-3531 lines, respectively (Delibes *et al*., 1997; Martín-Sánchez *et al*., 2003). Markers based on LRR sequences described as major determinants of the specificity of NBS-LRR resistance genes in plants (Meyers *et al*., 1999; Hammond-Kosack and Parker, 2003) are currently searched. This type of resistance genes includes the majority of the described resistance genes in plants (McHale *et al*., 2006). The strategy combines a PCR primer designed from the conserved NBS domain of these resistance genes and a primer from conserved domains of transposons reported to be present in high copy number within the *Triticeae* and commonly inserted near rich gene regions (Kalendar *et al*., 2004). The novel markers will be scored as co-dominant SNP-based PCR markers as reported by Moreno-Vázquez *et al*. (2003).

Pyramiding resistance genes

Pyramiding by MAS different resistance genes into a genotype support a most durable resistance to pathogens and pests (Pedersen and Leath, 1988). The combination of the CCN resistance genes described by our group (*Cre*2, *Cre*6 and *Cre*7), and also with other non-allelic resistance sources may be desirable to improve the maintenance of CCN nematode resistance in wheat. With that in mind, the efficiency of all *Cre* genes against Spanish pathotype Ha71 was firstly studied and then allelism tests carried out. Genes *Cre*3, *Cre*6 and *Cre*8 are ineffective for that pathotype. The extent to what Ha71 reproduction is inhibited, permits to rank *Cre*1 ≥ *Cre*4 ≥ *Cre*7>*Cre*5 ≥ *Cre*2>>*Cre*8>*Cre*3>*Cre*6 (Delibes *et al*., 1993; Romero *et al*., 1998; Ogbonnaya *et al*., 2001; Montes *et al*., 2003; and unpublished data). The genes *Cre*1, *Cre*4 and *Cre*5 are non-allelic to *Cre*2 and *Cre*7 and could be combined in the same genotype reducing the likelihood of virulent pathotypes emerging and increasing the durability of resistance (Delibes *et al*., 1993; Romero *et al*., 1998; unpublished data).

In a similar approach, 21 hexaploid wheat cultivars carrying different HF resistance genes from Uniform Hessian Fly Nursery (UHFN) were tested for resistance in infested fields in the SW of Spain. In general, the tested UHFN cultivars had a high resistance level to the biotype prevailing in Spain. Several of these cultivars were crossed with H-93-33 and TR-3531 resistant lines, in order to verify whether *H*27 and *H*30 described in our laboratory are allelic to any of the previously identified genes. *H*27 was demonstrated not to be allelic with respect to *H*3, *H*5, *H*9, *H*11, *H*12, *H*18 and *H*21, and that *H*30 is non-allelic with respect to *H*3, *H*6, *H*9, *H*11, *H*12, *H*13, *H*18 and *H*21 present in different UHFN wheat cultivars (Martín-Sánchez *et al*., 2003; and unpublished data). To detect individuals with two *H* genes (*H*27 or *H*30 and that of the other UHFN parents) F_2 plants are being studied with molecular markers linked to these RGs.

Release of varieties

In parallel to the described basic studies on resistance and defense genes, advanced lines incorporating resistance genes for CCN and/or HF are being developed. To this purpose, we are using lines H-93 or TR as donors of resistances and different commercial wheat cultivars with high yield and/or good quality as recurrent parents. The introduction of RGs from lines H-93-8 (gene *Cre*2), TR-3531 (genes *Cre*7 and *H*30) and H-93-33 (gene *H*27) into commercial wheat cultivars has been carried out by backcrossing and selection for the resistance itself or for markers linked to these RGs. Resulting lines with good performance for resistance and/or agronomic characteristics were subject to five parallel processes: 1) planting head to row in order to probe uniformity; 2) increase of the seed stock for quantitative and qualitative field tests; 3) field resistance tests; 4) MAS, whenever possible; 5) evaluation in different locations for production and traits related to quality.

After these studies the lines with the best overall performance were submitted to the Spanish Catalogue of Commercial Plant Varieties [*Registro de Variedades Comerciales*, http://www.mapa.es/app/RegVar/default.aspx?id=es, record numbers 20030279 (Victorino), 20040230 (Peñalón) and 20050190 (Mapeña)]. The main features of those selected lines are:

— «Victorino» (ID-2150) Spring bread wheat, developed from the backcross 'H-93-8/3*Rinconada'. It carries the *Cre*2 and is tolerant to *H. avenae.* High-yielding, mid-maturing, semidwarf cultivar, with moderate resistance to powdery mildew and leaf rust. Good baking quality. Best adapted to the southern and northeastern wheat growing regions of Spain. Already registered in the Spanish Catalogue of Commercial Plant Varieties (BOE, 2007a).

— «Peñalón» (T-2003) named in the honour to Manuel Alonso Peña. Spring bread wheat developed from the backcross 'TR-353/3*Osona//4*Cartaya'. It carries *Cre*7 and it is tolerant to *H. avenae.* Has been one of the top yielder lines in the national variety trial testing of Spain OEVV (see Fig. 2). Early maturing line, semidwarf, moderately resistant to powdery mildew and resistant to leaf rust. Best adapted to southern and northeastern wheat growing regions of Spain. Regular baking. Already registered in the Spanish Catalogue of Commercial Plant Varieties (BOE, 2007b).

— «Mapeña» (ID-2181) Spring bread wheat developed from the backcross 'TR353/Betres//Alcotan/3//Rinconada /4/3*Betres'. It carries *Cre*7 and it is tolerant to *H. avenae.* It is an early maturing line, semidwarf cultivar, good yielder, moderately susceptible to leaf rust. It is suitable for the baking industry.

Finally, advanced lines with HF resistance (genes *H*27 and *H*30) and/or notable agronomic characteristics have been developed. Thirty-four of these advanced lines are currently under field evaluation in five localities in Spain where the mosquito is naturally present.

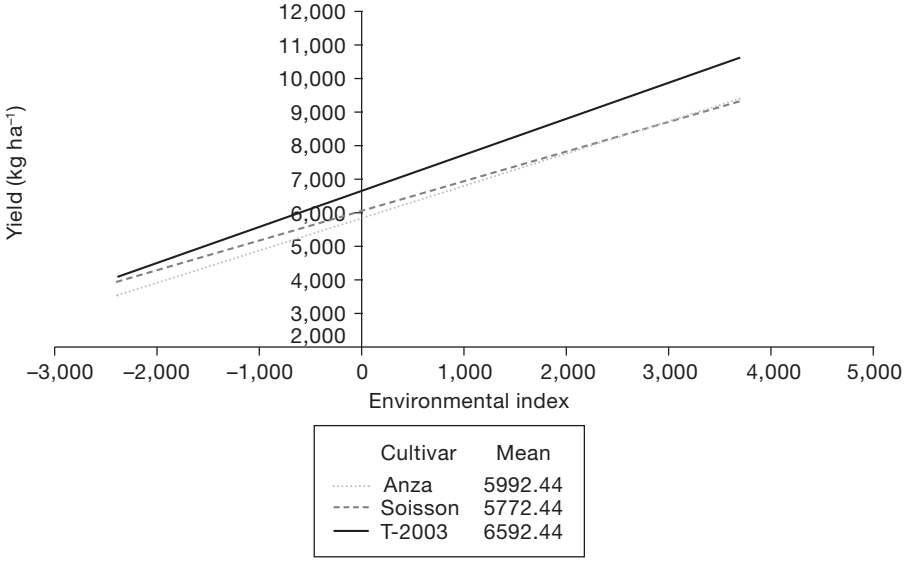

Figure 2. Regression between environmental index and grain production (kg ha^{-1}) under eight different growing conditions of advanced wheat line T-2003, carrying *Cre*7, in comparison to the official controls *T. aestivum* cvs. Anza and Soisson.

Subsequently, those lines homozygous for genes *H*27 or *H*30 with elevate and uniform yield and improved agronomic performance will be multiplied and submitted for registration at the Spanish *Registro de Variedades Comerciales*.

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