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Authors

Tranquilli, G
Lijavetzky, D
Muzzi, G
et al.

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G. Tranquilli · D. Lijavetzky · G. Muzzi · J. Dubcovsky

Genetic and physical characterization of grain texture-related loci in diploid wheat

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Abstract Endosperm texture, i.e. the hardness or softness of the grain, is an important quality criterion in cereals because it determines many grain end-use properties. Grain softness is the dominant trait and is mainly controlled by the *Ha* locus on the short arm of chromosome 5D in hexaploid bread wheat. Genes for puroindoline a (*Pina-D1*), puroindoline b (*Pinb-D1*), and grain softness related protein (*Gsp-D1*) have been shown to be linked to the *Ha* locus in different mapping populations and have been associated with the expression of grain softness. The study of the linkage relationships among these genes has been limited by the low level of polymorphism in the D genome of hexaploid *Triticum aestivum*. In the present study, a highly polymorphic *Triticum monococcum* mapping population was used to analyze linkage relationships among these three genes. *Gsp-A^m1* and *Pina-A^m1* were found to be completely linked and lie 0.14 cM distal to *Pinb-A^m1* in the distal region of the short arm of chromosome 5A^m. The tight genetic linkage among these three genes was paralleled by their physical proximity within a single 105-kb clone isolated from a *T. monococcum* bacterial artificial chromosome (BAC) library. A restriction map of this BAC clone showed that *Pina-A^m1* is located between *Pinb-A^m1* and *Gsp-A^m1*. Partial sequences of the *T. monococcum* genes showed a high degree of similarity with their *T. aestivum* counterparts ($\geq 94\%$). Marker-assisted selection strategies based on the tight linkage among *Ha*-related genes are discussed.

Key words Grain hardness · Grain-softness-related protein · Puroindoline · Wheat · Physical map

Introduction

Bread wheat (*T. aestivum* L.) is used to manufacture different products that require specific grain characteristics. Endosperm texture, i.e. the hardness or softness of the grain, is one of the most important quality criteria because it determines many grain end-use properties. Hard-textured grains require more grinding energy than soft-textured grains to reduce endosperm into flour, and during this milling process a larger number of starch granules become physically damaged. Damaged starch granules absorb more water than undamaged granules and consequently hard wheat flours have higher water absorption than soft wheat flours. Water absorption also influences crumb softness and the keeping characteristics of bread. Flours from hard wheats are preferred for breadmaking while flours from soft wheats are preferred for manufacturing cookies and cakes (Tippless et al. 1994).

Endosperm texture is strongly dependent on genetic factors, shows simple inheritance (Symes 1965) and is controlled primarily by a single locus on the short arm of chromosome 5D in hexaploid bread wheat (Mattern et al. 1973; Law et al. 1978). Though this main locus is referred as *Hardness* (*Ha*), softness is in fact the dominant trait. Differences in endosperm softness are mainly due to differences in the strength of the adhesion between the starch granule surface and the surrounding matrix (Pomeranz and Williams 1990). Examination of proteins from washed starch preparations has shown that a 15-kD starch-surface-associated protein is present at relatively low levels on hard wheat starches and at relatively high levels on soft wheat starches (Greenwell and Schofield 1986, Jolly et al. 1993). This association provides an indication, although not proof, of a causal role for this protein, named friabilin, in controlling endosperm texture. The

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G. Tranquilli · D. Lijavetzky · G. Muzzi · J. Dubcovsky (✉)
Department of Agronomy and Range Science,
University of California,
Davis, CA 95616-8515, USA
E-mail: jdubcovsky@ucdavis.edu; Fax: +1-530-7524361

G. Tranquilli,
Instituto de Recursos Biológicos,
INTA, Villa Udaondo, (1712) Castelar,
Buenos Aires, Argentina

accumulation of friabilin in the seed is dependent on the short arm of chromosome 5D, suggesting that this protein may be the product of the *Ha* locus and therefore represent a marker for grain-softness (Jolly et al. 1993).

Friabilin is not a single polypeptide but a composite of related proteins that include puroindoline-a (PinA), puroindoline-b (PinB), and the grain softness protein family *Gsp1*, which includes *Gsp-1a*, *Gsp-1b* and *Gsp-1c*. cDNAs encoding for these proteins have been characterized (Gautier et al. 1994; Rahman et al. 1994) and mapped on the distal part of chromosome 5DS completely linked to the *Ha* locus. The *Pina-1* and *Pinb-1* loci have been detected only on the 5D chromosome but homoeologous *Gsp-1* loci were detected on chromosomes 5A, 5B and 5D (Dubcovsky and Dvorak 1995; Jolly et al. 1996; Sourdille et al. 1996; Giroux and Morris 1997). Though linkage of puroindoline and *Gsp-D1* genes to the *Ha* locus in different mapping populations suggests that these genes should be linked, no estimate is available of the genetic distances among these three genes because the limited polymorphism on the D genome of *T. aestivum* has precluded their study in a common mapping population.

Since the *Ha*-related genes may act in conjunction on the expression of grain softness (Giroux and Morris 1997, 1998), a more precise understanding of the genetic linkage between these loci would be useful for designing strategies to manipulate wheat grain hardness. The goal of this work was to study the genetic linkage and physical organization of the *Ha*-related genes by using diploid wheat (*Triticum monococcum* L.). Partial sequences of the *Ha*-related genes from the A^m genome of *T. monococcum* were compared with those from the D genome of *T. aestivum*.

Materials and methods

Mapping populations

Two segregating F₂ populations of *T. monococcum* were used for mapping *Ha*-related genes on chromosome 5A^m. The first one included 355 individuals from a cross between a cultivated *T. monococcum* (DV92) and a wild *T. monococcum* ssp. *aegilopoides* (Link.) Thell. (G3116). The second one comprised 76 F₂ plants from a cross between wild *T. monococcum* ssp. *aegilopoides* accessions G2528 and G1777. Thus, in all, a total of 862 chromosomes was analyzed to determine the linkage relationships between the *Ha*-related genes. Linkage maps were constructed with the aid of the computer program Mapmaker/EXP 3.0 (Lander et al. 1987) using the Kosambi function.

Clones for the *Ha*-related genes

Clone *pGsp* for the grain softness-related protein was kindly supplied by S. Rahman (Rahman et al. 1994). Clone *pTa31*, which corresponds to the *Pina-D1* cDNA, was kindly provided by P. Joudrier (Sourdille et al. 1996). The clone for *Pinb-A^m1* was obtained by PCR amplification of *T. monococcum* DNA using primers developed by Gautier et al. (1994). The PCR product was

cloned into pCR2.1 vector using the TA Cloning Kit (Invitrogen), and designated *pTam19B2* to indicate its similarity to the original clone *pTa19B2* (Gautier et al. 1994).

Experimental procedures

Nuclear DNA was isolated from leaves of single F₂ plants and parental lines, following the procedure of Dvorak et al. (1988). Southern blotting and hybridization was performed as described by Dubcovsky et al. (1994). DNAs from DV92, G3116, G1777, G2528 were digested with eight restriction enzymes and screened for polymorphism. Restriction enzyme *Bam*HI was used to map the three *Ha*-related genes in the DV92 × G3116 population. In the G1777 × G2528 population *pTa31* was mapped with *Bgl*II and *pGsp* with *Eco*RI (*pTam19B2* was not polymorphic in this population).

Clones *pGsp* and *pTa31* were used to screen a *T. monococcum* BAC library (Lijavetzky et al. 1999). The single BAC clone detected with these two cDNAs was digested with the 8-bp cutters *Asc*I, *Fse*I, *Not*I, *Pac*I and *Pme*I. All possible double, triple and quadruple digestions were analyzed for those restriction enzymes for which one or more internal recognition sites were present within this BAC. Restriction fragments were separated by pulsed-field electrophoresis in 1% agarose gels (for 14 h at 12°C and 200 V, with a pulse time of 0.2–13 s). Southern blots were hybridized with [α -³²P]-labeled *pGsp*, *pTam19B2*, *pTa31* and BAC vector *pINDI-GO451*. This BAC vector was used to construct the *T. monococcum* BAC library and was kindly provided by Dr. H. Shizuya (California Institute of Technology, Pasadena).

To confirm the presence of the *Ha*-related genes within the *T. monococcum* BAC clone, DNA from the BAC was used as a template to amplify fragments from each of the *Ha*-related genes. Specific PCR primers were designed based on the sequences of *pTa31* (*Pina-D1*; Gautier et al. 1994) and *pGsp* clones (Rahman et al. 1994). The sense- and antisense-strand primers for the *Pina-1* gene were 5'-CCCTGTAGAGACAAAGCTAA-3' and 5'-TCAC-CAGTAATAGCCAATAGTG-3'; and for the *Gsp-1* gene were 5'-GCAAGCTCCACCGCGGATG-3' and 5'-CCAGTAATAT-CCGCTAGTGAT-3'. The primers and PCR conditions reported by Gautier et al. (1994) were used to amplify the *Pinb-A^m1* gene (5'-ATGAAGACCTTATTCCTCCTA-3' and 5'-TCACCAGTAA TAGCCACTAGGGAA-3'). Four nanograms of each PCR product was cloned into the pCR2.1 vector according to the protocols supplied with the TA Cloning Kit (Invitrogen). Each clone was sequenced on both strands using an ABI377 automatic sequencer (Applied Biosystems). DNA sequences of *Pina-A^m1* (EMBL accession No. AJ242715), *Pinb-A^m1* (EMBL accession No. AJ242716) and *Gsp-A^m1* (EMBL accession No. AJ242717) from *T. monococcum* were compared with published sequences from *T. aestivum* using BLASTN (Altschul et al. 1990).

Results

Genetic map

Different RFLP patterns were obtained when *pGsp*, *pTa31* and *pTam19B2* clones were hybridized to blots of digested DNA from the parental *T. monococcum* lines, suggesting that these sequences are not physically adjacent. Clones *pGsp* and *pTa31* hybridized with a single restriction fragment in most of the restriction digests analyzed. No recombination was detected between the main RFLP bands detected with these two clones among the 431 F₂ individuals of *T. monococcum* analyzed here. Complete linkage in 862 chromosomes indicates a maximum genetic distance between the *Gsp-A^m1* and

Pina-A^m1 genes of 0.3 cM with a 95% probability (Hanson 1959).

Clone pTam19B2 also hybridized with a single restriction fragment with most of the restriction enzymes analyzed. Only one plant, designated #241, showed recombination between the *Pinb-A^m1* gene and the completely linked *Gsp-A^m1*–*Pina-A^m1* genes. A genetic distance of 0.14 cM was estimated based on the recovery of one recombinant among the 359 F₂ plants analyzed with this clone.

In order to define the relative orientation of the *Ha*-related genes on the short arm of chromosome 5A^m, the flanking loci *XNor* and *Xmwig920* (Dubcovsky et al. 1996) were analyzed in 90 F₂ plants including #241. This plant was homozygous for the *Nor* and the *Gsp-A^m1*–*Pina-A^m1* genes, and heterozygous for the *Pinb-A^m1* gene and the proximal locus *Xmwig920*. Based on this result the most likely order of the *Ha*-related genes is that presented in Fig. 1, with the *Gsp-A^m1* and *Pina-A^m1* genes distal to the *Pinb-A^m1* gene (Fig. 1). The LOD score for this order was 2.4 units higher than for the alternative order *Xmwig920*–(*Gsp-A^m1*–*Pina-A^m1*)–*Pinb-A^m1*–*XNor*, indicating that the latter order is 250 times more unlikely than the order presented in Fig. 1.

Physical map

Four high-density filters (bearing 73,728 clones) representing 1.6 genome equivalents of *T. monococcum* were hybridized with the clones pGsp and pTa31. Only one BAC clone, 109N23, hybridized with both clones.

This 105-kb BAC clone showed four *Asc*I restriction sites, three *Not*I restriction sites, two *Pac*I restriction sites and three *Pme*I restriction sites. No *Fse*I restriction site was present in this BAC clone and this enzyme was not used for further analyses. Sizes of the restriction fragments generated by single, double, triple, and quadruple digestions were used to construct the restriction map shown in Fig. 1. Most fragments were ordered unambiguously, with the exception of a group of three adjacent *Asc*I fragments (6.5 kb, 8.2 kb and 9.2 kb fragments, Fig. 1). The restriction map in Fig. 1 was confirmed by hybridization with four different clones.

Hybridization with the [α -³²P]-labeled vector p451 IndigoBAC was useful not only in identifying the restriction fragments that included vector sequences, but also to confirm the size of the *T. monococcum* fragments adjacent to the vector. This was possible because small segments of the vector remain attached to the adjacent insert after digestion with the restriction enzyme *Not*I, producing a detectable hybridization signal.

Hybridization with clones pGsp, pTa31 and pTam19B2 indicated that all these genes were located within BAC clone 109N23. Clone pGsp hybridized with a 13.3-kb *Pme*I fragment, clone pTa31 hybridized to a 6.6-kb *Asc*I–*Pme*I fragment and pTam19B2 to a 29-kb *Pac*I–*Pme*I fragment (Fig. 1). The integration of this information in Fig. 1 showed that the *Pina-A^m1* gene was located between the genes *Pinb-A^m1* and the *Gsp-A^m1*.

Sequence analysis

Partial sequences of the *Gsp-A^m1* (406 bp), *Pina-A^m1* (331 bp) and *Pinb-A^m1* (447 bp) genes from *T. monococcum* were compared with the corresponding sequences from *T. aestivum* using BLASTN (Altschul et al. 1990). The identity values were 98% for *Pina-I* sequences, 94% for *Pinb-I* sequences, and 95%, 94%, and 97% for *Gsp-A^m1* with *Gsp-1a*, *Gsp-1b*, *Gsp-1c*, respectively. The higher homology of *Gsp-A^m1* to *Gsp-1c* relative to the other *Gsp-I* sequences suggests that *Gsp-*

Fig. 1 Physical and genetic maps of the *Ha*-related genes region on the short arm of wheat chromosome 5A^mS. The physical map of the BAC clone 109N23 is based on digestion with all possible combinations of the restriction enzymes *Not*I (N), *Asc*I (As), *Pac*I (Pc), and *Pme*I (Pm). Restriction fragments containing the *Gsp-A^m1*, *Pina-A^m1*, and *Pinb-A^m1* genes were identified by hybridization and are indicated by filled, stippled, and hatched areas, respectively. The genetic distances from *Xmwig920* and *XNor* to the *Ha*-related genes are from Dubcovsky and Dvorak (1995). The locus *Xmwig920* is approximately 40 cM from the centromere and *XNor* is the most distal locus

Ic derives from the A genome of wheat. These results confirmed the presence of *Gsp-A^m1*, *Pina-A^m1* and *Pinb-A^m1* genes within BAC clone 109N23.

Discussion

In hexaploid wheat the *Pina-1* and *Pinb-1* genes are present only on chromosome 5D. However, in *T. monococcum* they are both present on chromosome 5A^m, indicating that their absence in *T. aestivum* A and B genomes is most likely to be due to deletions. The loci *Pina-D1* (Sourdille et al. 1996) and *Pinb-D1* (Giroux and Morris 1997) were previously mapped to the distal region of chromosome 5DS in different mapping populations. The grain softness-related gene *Gsp-1* was also mapped on the same region of chromosome 5D of *T. aestivum* and *Aegilops tauschii* (Dubcovsky and Dvorak 1995; Jolly et al. 1996) and chromosome 5A^m of *T. monococcum* (Dubcovsky et al. 1996). This indirect evidence for linkage among the three *Ha*-related genes was confirmed here by demonstrating that these three genes are tightly linked in the distal region of the short arm of chromosome 5A^m in a single segregating population.

The tight linkage of the *Ha*-related genes can be explained by their physical proximity within a single 105-kb BAC clone. The observed distances among these three genes are smaller than the distances expected from a uniform distribution of genes along the wheat genome. Assuming that the total number of genes in diploid *T. monococcum* is similar to the total number of genes in *Arabidopsis thaliana* (≈21,000, Bevan et al. 1998), and considering that the haploid genome size of *T. monococcum* is 5,600 Mb (Furata et al. 1986), the expected average distance between genes is 270-kb. Therefore, the estimated distance between these three genes in the 109N23 BAC clone is between seven and fifteen times smaller than expected from the uniform distribution hypothesis, depending on the actual position of the genes within the identified fragments. This is a conservative estimate because it is still not known if additional genes are present within the 109N23 BAC clone. Other examples of gene-rich regions in the large genomes of wheat and barley genomes have been reported before (Gill et al. 1996; Panstruga et al. 1998).

It has been suggested that these gene-rich regions in wheat are also recombination-rich areas (Gill et al. 1996). This seems to be the case for the 109N23 BAC clone, where *Pina-A^m1* and *Pinb-A^m1* loci, separated by no more than 36 kb, were 0.14 cM apart. This genetic distance is 20 times larger than expected from an even distribution of recombination along the 1100 cM of the *T. monococcum* chromosomes (Dubcovsky et al. 1996). However, it should be pointed out that the sample size of the mapping populations used here is not large enough to declare this difference significant ($P \geq 0.05$).

It is tempting to speculate that *Ha*-related genes on chromosome 5D of *T. aestivum* will have a similar organization to that described here for chromosome

5A^m. This hypothesis is based on the high degree of similarity found between *Ha*-related gene sequences from *T. monococcum* and their *T. aestivum* counterparts, the similar chromosome location of the *Ha*-related genes, and the colinearity between the short arms of chromosomes 5A^m and 5D (Dubcovsky and Dvorak 1995). However, there are known examples for disturbed colinearity within the Triticeae and experimental evidence is required to confirm the microcolinearity of the A^m and D genomes in the *Ha* region.

If linkage of the *Ha*-related genes in the D genome is similar to that observed here for the A^m genome, variation in any of these genes can be used as a marker to trace a particular combination of *Ha*-related alleles. One possible tool for tracing these combinations is the set of specific PCR primers designed by Giroux and Morris (1997, 1998) to discriminate a glycine-to-serine point mutation in the *Pinb-D1* gene. However, the discriminatory power of these primers is based on a single base pair difference at the 3' end of the primers, and occasional false positives are observed when PCR conditions are not perfectly optimized. To avoid this problem we developed a codominant CAPS marker (cleavage amplified polymorphic sequence; Jarvis et al. 1994) based on the same point mutation. After amplification of a 447-bp segment of *Pinb-D1* using the external primers designed by Gautier et al. (1994) (see Materials and methods), the PCR product is digested with the restriction enzyme *Bsr*BI. This enzyme recognizes the sequence with the glycine-to-serine mutation but not the sequence without the mutation present in all soft varieties.

Based on the tight linkage demonstrated here between the puroindoline genes, this CAPS marker for the *Pinb-D1* gene can be also used to tag the null mutant in the *Pina-D1* locus. This null *Pina-D1* variant, observed in many hard varieties, is always associated with a *Pinb-D1* allele without the glycine-to-serine mutation (Giroux and Morris 1997, 1998). In these hard varieties the PCR product will not be digestible with the restriction enzyme *Bsr*BI, just as with a soft variety. All other hard varieties without the null *Pina-D1* have the glycine-to-serine mutation; thus, the PCR product will be digestible with *Bsr*BI. Preliminary results indicate that bread wheat varieties with the null *Pina-D1* deletion have harder grains than varieties with the glycine-to-serine mutation (Giroux et al. 1998; Morris et al. 1999) and, therefore, this CAPS marker may be useful for making small adjustments in the level of hardness of hard wheat varieties.

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