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Variation for grain protein content and identification of QTLs by molecular markers in tetraploid wheats

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SUMMARY – The wild tetraploid wheat *T. turgidum* var. *dicoccoides* shows particular promise as a donor of useful variation for several traits, including grain protein quantity and quality. The effectiveness of the inbred backcross method to identify quantitative trait loci (QTL) and for introgressing only the desired trait from wild populations was examined. A set of 92 backcross inbred lines (BIL) was developed from a cross between the durum wheat cv. Latino and the accession MG29896 of the var. *dicoccoides* and tested for grain protein content in three locations of South-Italy over two years. Variation among the BIL population was observed and superior BILs for protein content were identified. Putative QTL on different chromosomes were detected by association with molecular markers.

Key words: Wheat, germplasm, grain protein content, molecular markers.

RESUME – *"Variation de la teneur en protéine du grain et identification de QTL par marqueurs moléculaires chez les blés tétraploïdes". Le blé dur sauvage* T. turgidum *var.* dicoccoides *est particulièrement indiqué comme donneur de variations utiles pour plusieurs caractères, y compris ceux pour la qualité et la quantité de protéines du caryopse. L'efficacité de la méthode des lignées inbred de recroisement pour identifier les QTL (gènes à effet quantitatif) et pour incorporer seulement les caractères désirés à partir des populations sauvages a été examinée. Un groupe de 92 BIL (lignées inbred de recroisement) a été développé à partir d'un croisement entre le blé dur cv. Latino et l'accession MG29896 de la var.* dicoccoides *et le contenu protéique de ce groupe de BIL a été évalué dans trois localités de l'Italie méridionale pendant deux années. Les variations dans la population BIL ont été observées et les BIL supérieurs pour le contenu des protéines ont été identifiés. Les QTL sur chromosomes différents ont été relevés par association avec marqueurs moléculaires.*

Mots-clés : *Blé, germoplasme, contenu des protéines du caryopse, marqueurs moléculaires.*

Introduction

In the years ahead, wheat, perhaps more than other cereals, can be expected to assume greater importance as a source of protein for much of the world's increasing population. Seed storage protein content of bread and durum wheats has an important effect both on nutritional value as well as on bread-baking and pasta-making characteristics, respectively. Under normal cropping conditions, wheats show a low protein content, ranging between 10 and 14%. During the past 20 years, a rise in grain protein content (GPC) has been achieved, mainly through increased nitrogen fertilization. Any genetic improvement in protein content has been restricted by the negative correlation between productivity and protein content found in segregating populations in all cereals (Cox *et al.,* 1985). Thus, in the selection of higher yielding genotypes, it has been easier to increase carbohydrate than protein. The release of the cultivar Karl 92 and Jagger (Sears *et al.,* 1997a,b) indicates, however, that development of semidwarf cultivars with high grain yield and high grain protein concentration is possible.

When inheritance studies have been done, GPC has generally exhibited continuous variation due to polygenic control and the influence of environmental factors, mainly nitrogen and water availability, temperature and light intensity. An extensive review by Konzak (1977) summarizes reports indicating that the factors influencing protein concentration in cultivars are located on all chromosomes. More recent investigations on the wild tetraploid wheat var. *dicoccoides* have located quantitative trait loci (QTLs) for GPC on the chromosomes of groups 5 and 7, and on 1AS and 1BS (Levy and Feldman, 1989), on chromosomes 2A, 3A, 6A, 3B, 4B, 6B and 7B (Joppa and Cantrell, 1990), and on chromosome arms 4BS, 5AL, 6AS, 6BS and 7BS (Blanco *et al.,* 1996). Since the beginning of the 1990s, molecular markers, such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD),

amplified fragment length polymorphism (AFLP) and microsatellites have brought a new tool to enhance the genetic analysis of quantitative traits of crop species (see Paterson, 1998, for a review). In wheat, molecular markers have been already utilized to identify several QTLs involved in the expression of complex traits, such as resistance to preharvest sprouting (Anderson *et al.,* 1993), drought induced ABA production (Quarrie *et al.,* 1994), fertility restoration (Ma and Sorrells, 1995), seed protein content (Blanco *et al.,* 1996; Joppa *et al.,* 1997), kernel hardness (Sourdille *et al.,* 1996), sedimentation volume (Blanco *et al.,* 1998), flour colour (Parker *et al.,* 1998) and flour viscosity (Udall *et al.,* 1999).

The lack of sufficient genetic variation within the cultivated wheats has often limited the ability of plant breeders to improve GPC. An interesting approach for increasing wheat protein content lies in the use of related *Triticum* and *Aegilops* species, particularly the progenitor of durum wheat, *T. turgidum* var. *dicoccoides* (Avivi, 1979; Law *et al.,* 1984; Levy and Feldman, 1989; Blanco *et al.,* 1990). The wild wheat germplasm, however, contains both favourable traits and many commercially unacceptable characters such as low yield, fragile spikes, lodging susceptibility. In early generations of traditional method of wheat breeding (i.e. pedigree, bulk breeding method) from crossing wild and cultivated wheats, plants are segregating for many agronomically important traits that may interact with the trait being introgressed and confound measurements. The inbred backcross method (Wehrhahn and Allard, 1965; Bliss, 1982) produces backcross inbred lines (BIL) that can be tested in replicated trials prior to selection. Recently, some breeding programs have adopted advanced backcross populations as a method for identification and introgression of useful genes from wild relatives or unadapted germplasm with the potential to improve the agronomic performance of elite cultivated lines (Tanksley and Nelson, 1996; Fulton *et al.,* 1997; Bernacchi *et al.,* 1998). The potential benefits of these lines versus more balanced populations (e.g. F_2, F_3, BC_1) depend on the possibility of reducing the introgressed chromosomal regions as well as of fine mapping loci linked with quantitative traits. Epistatic QTLs or QTLs with gene actions ranging from recessive to additive can be detected in the backcross inbred lines and each QTL is treated as single Mendelian factor. The inbred backcross method has been employed successfully to introgress major alleles contributing to quantitatively inherited traits from exotic germplasm into adapted breeding lines in common bean, *Cucumis sativa*, spring rape, tomato, common wheat.

The present investigation was initiated with the objective to introgress alleles for GPC from var. *dicoccoides* into more adapted and agronomically acceptable wheat germplasm by means of the inbred backcross method. In the present paper we report results concerning the identification of superior BILs for GPC, the estimates of genetic variance, heritability and correlation between GPC and grain yield per spike, the identification of molecular markers associated to GPC to be used in marker-assisted selection.

Materials and methods

Population development. A backcross inbred population was produced following the method described by Bliss (1982). The recurrent parent, the durum wheat cv. Latino, is a semidwarf high-yielding commercial cultivar with poor grain quality characteristics. The donor parent was the wild tetraploid wheat *Triticum turgidum* var. *dicoccoides* accession MG29896 and was obtained from the Institute of Germplasm, CNR, Bari (Italy). One hundred and ten plants were backcrossed each backcross generation to the recurrent parent prior to advancement of each backcross line by self-fertilization and single-seed descent for seven generations, resulting in one population of backcross inbred lines (BIL). No intentional selection was imposed during population development, but some lines were lost during backcrossing and self-fertilization program. The BC_3F_7 generation contained 92 BILs, which gave sufficient seed for use in replicated trials.

Field trials. The BIL population was grown in a randomized complete block design consisting of four blocks at three locations in southern Italy (Gaudiano, Foggia and Valenzano) in 1997 and 1998. Each block contained one plot of each of the 92 BIL, one plot of accession MG29896, and three plots of the recurrent parent cv. Latino. The trial conducted at Gaudiano in 1997 contained 87 BILs. Each plot contained 10 plants spaced 10 cm within the row and 30 cm between the rows. During the growing season, standard cultural practices were used.

Trait evaluation. Grain yield per spike measures from eight main spikes per plot (from plants in the middle of the rows) were averaged to give a mean value per line to be used for statistical analysis. Protein percentage was measured on a 2 g sample of whole-meal flour by near-infrared reflectance spectroscopy and expressed on a dry weight basis.

Morphological markers. Genetic polymorphism concerned six morphological markers, including glaucousness (*W1*), glume colour (*Bg*), tenacious glumes (*Tg*), hairy glumes (*Hg*), response to gibberellic acid (*Gai1*) and red coleoptile (*Rc1*) (see catalogue by McIntosh *et al.,* 1998).

Seed storage protein markers. Polymorphisms for gliadin and HMW-glutenin components of grain storage proteins were identified in the BIL population. Total seed protein extraction and sodium dodecyl sulphate polyacrylamide gel elctrophoresis (SDS-PAGE) on 10% gels were carried out according to Payne *et al.* (1981). Monomeric prolamins were extracted from crushed single grains with 1.5M dimethylformamide at 1:5 w/v ratio; after centrifugation (15 min at 10,000 x g), the clear supernatant was used for electrophoretic separation, as described by Lafiandra and Kasarda (1985).

Microsatellites analysis. DNA extraction from frozen-dried leaves was performed as described by Sharp *et al.* (1988). Eighty primer pairs of wheat microsatellites (GWM) representing chromosomes of the A- and B-genomes of hexaploid wheat were chosen for analysis. The development of the microsatellite markers was described by Roder *et al.* (1995). WMS designation, primer sequences, chromosome location of the amplified loci and the annealing temperature for most of primers employed in this study are presented by Roder *et al.* (1998). PCR reactions and fragment detection were performed ad described by Roder *et al.* (1995).

Data analysis. Each year-location combination was treated as an environment in the subsequent statistical analyses. Standard procedures for analysis of variance were used to partition the total variance due to GPC into environments, lines, line-environment interaction, and error, by taking both factors as random. Dunnett's test was used to compare the mean of each BIL versus the mean of the recurrent parent cv. Latino within environments and across environments. Phenotypic differences are reported when showing a significant level of $P \leq 0.05$. Pearson phenotypic correlation coefficients were calculated between GPC and grain yield per spike in each environment and across environments. For the genetic analysis, BILs were treated as inbred lines, and line mean broad-sense heritability estimates for BILs were calculated according to Hallauer and Miranda (1988).

Results and discussion

BIL performance

Grain protein content was evaluated in six replicated trials conducted in three locations in southern Italy in 1997 and 1998. The analysis of variance revealed highly significant differences ($P \leq 0.001$) among BILs in each of the six environments (data not shown). The parental lines had significantly different GPC values in each environment, the var. *dicoccoides* having always a higher value than the cultivated parent Latino. Large segregation was observed in each of the six trials, with phenotypic means of BILs being normally distributed without significant skewness or kurtosis. Differences in mean values and variances of parental lines and BIL population were observed among the trials conducted in different years and locations presumably due to the different climatic conditions.

Estimates of heritability (line mean basis) of GPC were moderately high and ranging from 37.7% to 71.6% in the six environments. The value 36.2% was observed across environments. The environments, genotypes and environment x line interaction items were all significant in the combined analysis across environments (Table 1). Grain protein content, as expected, was negatively correlated with grain yield per spike across environments ($r = -0.23^{***}$). Minimal differences were observed among phenotypic correlations in each environment.

Results from Dunnett's pairwise tests of GPC indicated that out of 87 BILs tested in the six environments 9 lines had significantly greater GPC measurements than Latino (10.3%). One BIL had more than 0.9 GPC percentage units ($P \leq 0.05$) and 8 BIL more than 1.1 GPC percentage units ($P \leq 0.01$) than the cultivated parent. Notably three lines (BIL-18, BIL-69, BIL-85) had more than 16% GPC across environments (Table 2). The performance of the selected BILs, relative to the recurrent parent control, was variable across environments. Only the BIL-85 showed a GPC improvement over the control in all six environments tested; one line (BIL-69) had an improved GPC in five environments and four lines (BIL-15, BIL-18, BIL-48, BIL-68) had an improved GPC in three environments over the control. It is likely that some QTL-alleles interact with the environment resulting in the variable performance of the BILs across environments. Indeed, the ANOVA showed that the line x environment interaction was highly significant.

The significant phenotypic differences should be due to introgressed wild DNA segments. A comparison of the trait distributions of the BIL population, the selected BC_3 lines and the recurrent control Latino, provides a first indication of the phenotypic gains affected by the targeted wild alleles (Fig. 1).

Table 1. Analysis of variance of grain protein content (%) of a backcross inbred population derived from the cross *Triticum turgidum* var. *durum* cv. Latino x *T. turgidum* var. *dicoccoides* acc. MG29896. Data from a randomized complete block design of BC3F7 and BC3F8 plants grown in 1997 and 1998 in field plot at Valenzano (BA), Gaudiano (PT) and Foggia

Source of variation	Degree of freedom	Mean square	C.V. (%)	σ_G^2	h_B^2
Environments	5	1341.765***	5.5	0.281	36.2
Block (environments)	18	9.095			
Lines	90	8.593***			
Environment x lines	450	1.844***			
Error	1620	0.685			
Total	2183				

***Significant at 0.001 probability level.

Table 2. Grain protein content (%) of selected “Latino x dicoccoides” backcross inbred lines and the recurrent parent “Latino” over six environments

Line	Environments†						Across environments
	1	2	3	4	5	6	
BIL-15	16.8	16.7*	17.3	15.1*	13.9**	16.5	16.0**
BIL-18	17.6**	16.3	18.0**	16.9**	12.0	16.5	16.2**
BIL-29	16.6	16.1	17.0	16.1**	11.9	16.4	15.7*
BIL-48	18.2**	17.0**	18.5**	14.4	12.0	15.9	16.0**
BIL-60	19.0**	16.4	17.4	13.7	12.4	16.8	15.9**
BIL-68	16.5	17.1**	17.4	15.9**	12.9**	16.5	16.0**
BIL-69	17.4*	18.0**	19.3**	15.6**	12.8**	16.9	16.6**
BIL-77	16.7	17.6**	19.8**	14.1	11.7	15.6	15.9**
BIL-85	17.5*	18.0**	18.0**	17.4**	13.3**	18.0**	17.0**
LATINO	15.1	15.5	17.1	13.0	11.6	16.7	14.7
t Dunnett (0.05P)	2.14	1.23	0.42	2.04	0.94	0.62	0.94
t Dunnett (0.01P)	2.54	1.47	0.50	2.43	1.12	0.73	1.12

†Testing environments: 1 = Valenzano 1997, 2 = Gaudiano 1997, 3 = Foggia 1997, 4 = Valenzano 1998, 5 = Gaudiano 1998, 6 = Foggia 1998.

*,**Significant at 0.05 and 0.01 probability levels, respectively.

Identification of linked genetic and molecular markers

Mapping QTLs in simple BC or F_2 populations shows an important feature that limits their usefulness: evaluation of quantitative effects in a given marker-monitored chromosomal region takes place in the context of a variable genetic background, requiring therefore that large populations be handled for the marker assessments. These drawbacks can be overcome by the use of backcross inbred lines initially developed by Wehrhahn and Allard (1965) for the study of polygenic traits. With the objective to map protein content QTLs, a series of 92 BILs was generated from crossing the cultivated durum wheat Latino to the var. *dicoccoides*, acc. MG29896. After 3 backcross generations to the recurrent parent, the BIL genome consists almost exclusively (93.7%) of that of the recurrent parent. However, small chromosomal segments of the donor parent persist. The products of introgression will be a series of near-isogenic lines which are identical to each other and to the recurrent parent (Latino) except for single (or very few) small donor segments. The

BILs selected for high GPC, significantly different from Latino at $P \leq 0.05$ or $P \leq 0.01$, can be therefore considered as single gene-deviate from the recurrent parent. Markers located outside a segment with a target gene will exhibit identical pattern between the BILs and the recurrent parent, while markers located inside the segment may exhibit one or more polymorphisms. In order to identify genetic and molecular markers located near grain protein content QTLs, a total of 90 markers (6 morphological, 6 proteic and 78 microsatellite markers) were at first examined in the parental lines Latino and var. *dicoccoides*. The six morphological and the six proteic markers were found to be polymorphic. Out of 78 microsatellite markers, 48 (60%) were polymorphic between the parental lines and then examined in PCR reactions with genomic DNA of the nine BILs selected for their higher GPC than Latino (Fig. 2). Most of the tested microsatellite markers produced identical amplification patterns with DNA from the recurrent parent. Some markers, however, showed an identical amplification pattern in the BILs and the high GPC donor parent. So far introgression of 18 segments of the var. *dicoccoides* donor genome located on 8 different chromosomes were detected. After 3 backcrosses, however, the BILs retain yet 6.25% of the donor parent and then they can deviate for quite a few donor segments different from the introgressed segments with the target QTL.

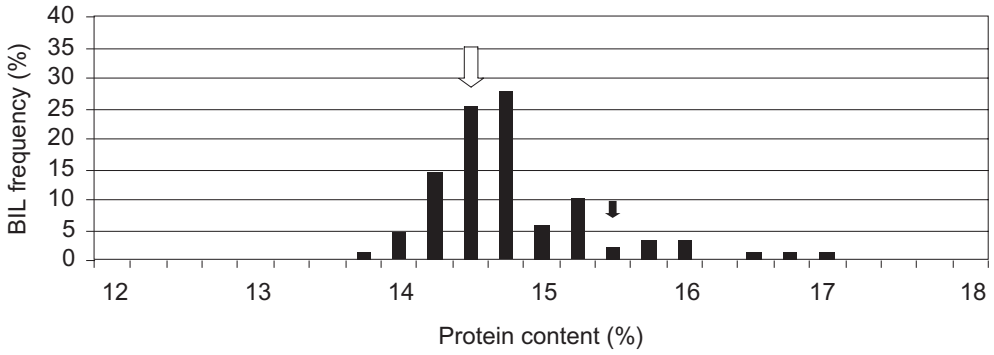

Fig. 1. Frequency distribution for grain protein content measured in the "Latino x *dicoccoides*" backcross inbred population. Histograms include observation from six environments. The mean value of the recurrent control, cv. Latino, is indicated by a large arrow. The small black arrow indicates the minimum level of the BILs that were significantly different from Latino at $P \leq 0.05$.

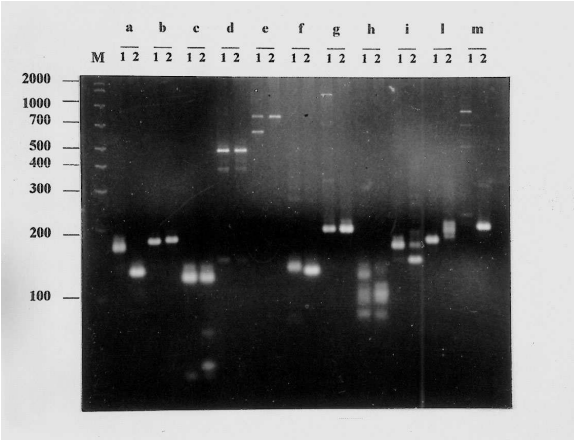

Fig. 2. Amplification of DNA from durum wheat cv. Latino (1) and var. *dicoccoides* acc. MG29896 (2) with eleven primer sets of microsatellites GWM5 (a), GWM18 (b), GWM33 (c), GWM43 (d), GWM174 (e), GWM108 (f), GWM126 (g), GWM132 (h), GWM148 (i), GWM160 (l), GWM162 (m). DNA size markers are shown in lane M with the size of the marker bands given in base pairs.

In order to distinguish the "false positives", 21 random lines of the 92 BIL series were examined with the 18 markers found to be polymorphic in the selected 9 BILs and the recurrent parent Latino. Sixteen markers were found to be also polymorphic in the random lines and Latino thus indicating that they

detected introgressed segments not involved in the expression of GPC loci. The results obtained on two high GPC lines (BIL-77 and BIL-85) indicate that two different donor segments, localized on the chromosome arms 1AS and 3BL and detected by the markers *Gli-A1* and *Xgwm299*, respectively, are associated to protein content QTLs. Such high GPC lines were backcrossed to Latino and the segregating BC_4F_2 generations will be again examined with markers and for GPC to confirm the molecular mapping of protein content loci. The number of putative QTLs described so far should be considered conservative as the molecular genetic map is incomplete and there are several gaps in the existing linkage map.

Mapping loci for GPC by genetic and molecular markers should enable the obtention of near-isogenic lines (NILs) in which the individual effects of each QTL can be examined in detail (Paterson *et al.,* 1990). In particular, markers can be used to develop lines that are homozygous for all the putative GPC loci except one, which is then expected to segregate as a Mendelian factor. In this way, individual effects could be examined without confounding variation due to other putative QTLs. The identification of molecular markers linked to GPC may potentially accelerate wheat breeding since the selection of superior plants can be carried out by genotype rather than phenotype. Moreover these NILs could also be useful to conduct physiological studies aimed at investigating the potential mechanisms leading to high GPC, to fine-map the position of each QTL, and eventually to map-based cloning.

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