Title

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Journal

Genome, 47(5)

ISSN

0831-2796

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Publication Date

2004-10-01

DOI

10.1139/g04-033

Peer reviewed

PCR identification of durum wheat BAC clones containing genes coding for carotenoid biosynthesis enzymes and their chromosome localization

A. Cenci, S. Somma, N. Chantret, J. Dubcovsky, and A. Blanco

Abstract: Carotenoids are essential components in all plants. Their accumulation in wheat seed determines the endosperm colour, which is an important quality trait in wheat. In this study, we report the isolation of BAC clones containing genes coding for three different enzymes of the carotenoid biosynthesis pathway: phytoene synthase (PSY), phytoene desaturase (PDS), and ζ-carotene desaturase (ZDS). Primers were designed on the basis of wheat ESTs similar to the sequences of these three genes in other species, and used to screen a BAC library from *Triticum turgidum* var. *durum* (2*n* = 28, genomes AABB). Eight, six, and nine 384-well plates containing at least one positive clone were found for PSY, PDS, and ZDS, respectively. BACs selected for each of these genes were then divided in two groups corresponding to the A and B genomes of tetraploid wheat, based on differences in the length of the PCR amplification products, conformation-sensitive gel electrophoresis (CSGE), or cleavage amplification polymorphisms. Positive clones were then assigned to chromosomes using a set of D genome substitution lines in *T. turgidum* var. *durum* 'Langdon'. PSY clones were localized on chromosomes 5A and 5B, PDS on chromosomes 4A and 4B, and ZDS on chromosomes 2A and 2B. The strategies used for the PCR screening of large BAC libraries and for the differentiation of BAC clones from different genomes in a polyploid species are discussed.

Key words: wheat, carotenoid biosynthesis, BAC.

Résumé : Les caroténoïdes sont des composants essentiels chez les plantes. Leur accumulation dans les grains de blé détermine la couleur de l'endosperme qui est un critère de la qualité important pour le blé. Dans cette étude, nous présentons l'isolement de clones BAC contenant les gènes codant pour trois enzymes différentes de la chaîne de biosynthèse des caroténoïdes: la phytoène synthase (PSY), la phytoène désaturase (PDS) et la ζ-carotène désaturase (ZDS). Des amorces ont été définies à partir des séquences d'EST de blé similaires aux gènes codant pour ces enzymes disponibles chez d'autres espèces, et utilisées pour cribler une banque BAC de l'espèce *Triticum turgidum* var. *durum* (2*n* = 28, génomes AABB). Huit, six et neuf plaques de 384 puits contenant au moins un clone positif ont été trouvées respectivement pour la PSY, la PDS et la ZDS. Les BACs sélectionnés pour chacun de ces gènes ont pu être divisés en deux groupes correspondant aux génomes A et B du blé tétraploïde, en se basant sur les différences de taille des produits d'amplification PCR, sur les différences de conformation entre les fragments amplifiés révélés par l'électrophorèse (CSGE) ou sur le polymorphisme de restriction des fragments amplifiés. Les clones positifs ont ensuite été assignés aux chromosomes en utilisant un jeu de lignées de substitution par le génome D dans le cultivar *T. turgidum* var. *durum* 'Langdon'. Les clones positifs pour la PSY ont été localisés sur les chromosomes 5A et 5B, ceux pour la PDS sur les chromosomes 4A et 4B, et ceux pour la ZDS sur les chromosomes 2A et 2B. Les stratégies utilisées pour le criblage par PCR d'une banque BAC de grande taille et pour différencier les clones BAC provenant de différents génomes d'une espèce polyploïde sont discutés.

Mots clés : blé, biosynthèse de caroténoïdes, BAC.

Received 21 May 2003. Accepted 16 March 2004. Published on the NRC Research Press Web site at http://genome.nrc.ca on 20 August 2004.

Corresponding Editor: J.P. Gustafson.

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 doi: 10.1139/G04-033

Introduction

Carotenoids are essential components of the photosynthetic system in all plants, mainly harvesting light for photosynthesis and dissipating excess light energy; they are also colouring agents and precursors for abscisic acid biosynthesis. In human and animal diets, carotenoids play an important protective role as antioxidants. In addition, β-carotene is a precursor of essential vitamin A. Carotenoids provide colours (yellow, orange, and red) that have economic relevance in many crops and ornamental plants and therefore their biosynthesis pathway has been extensively studied (Cunningham and Gantt 1998). Briefly, the process involves the synthesis of a 40 carbon atom chain (C40) backbone, its desaturation, cyclization, and subsequent modifications (e.g., hydroxylation and epoxidation). The two first steps are common for all carotenoid pathways, whereas cyclization can produce β or ε rings having different properties. Phytoene synthase (PSY) catalyses the reaction making phytoene (C40) from two molecules of geranyl-geranyl pyrophosphate (C20); phytoene desaturase (PDS) catalyses the double desaturation at the 11–12 and 11′–12′ symmetrical positions of phytoene producing ζ-carotene; and ζ-carotene desaturase (ZDS) catalyses the reaction producing lycopene by desaturation of 7–8 and 7′–8′ symmetrical positions of ζ-carotene. Double lycopene cyclization can produce β-, ε-, or α-carotene, having β–β, ε–ε, and β–ε rings, respectively. Subsequent modifications transform α-, β-, and ε-carotene into lutein or other xanthophylles.

Carotenoid accumulation in wheat seeds confers yellow colour to the flour or semolina. This is a negative trait for the breadmaking industry because of the consumer preference for white bread. On the contrary, it is a very valuable trait for the pasta industry because yellow macaroni are preferred by the market. Genetic studies showed that wheat endosperm yellow colour is highly heritable (67–90%) and under poligenic control (Parker et al. 1998; Elouafi et al. 2002; A. Blanco, unpublished data). Quantitative trait loci (QTL) for endosperm yellow colour accounting for a large proportion of the genetic variation (50%–60%) have been mapped on chromosome arms 7AL and 7BL. Smaller QTLs were detected on chromosomes 2A, 3A, 5A, and 5B (Parker et al. 1998; Elouafi et al. 2002; A. Blanco, unpublished data). However, the genes responsible for the differences in carotenoid accumulation in the wheat grain have not been identified yet.

Genes involved in the carotenoid biosynthesis pathway are good candidates to explain some of the differences in endosperm colour among cultivars. To characterize these genes we used a recently developed bacterial artificial chromosomes (BAC) library of *Triticum turgidum* (L.) Thell. var. *durum* (Korn.) Thell. ($2n = 4x = 28$, genomes AABB) (Cenci et al. 2003). This BAC library has a 5× genome coverage providing a high probability of recovering the targeted genes. In the present paper, we describe the PCR strategy used to identify BAC clones containing the *PSY*, *PDS*, and *ZDS* genes involved in the carotenoid biosynthesis pathway, and the assignment of these BACs to wheat chromosomes.

Materials and methods

Plant and BAC material

The plant material used in this study included the tetraploid wheat *Triticum turgidum* var. *durum* 'Langdon' (LDN), the set of substitution lines of each of the 14 chromosomes of LDN replaced by the Triticum aestivum 'Chinese Spring' D genome homoeologous chromosome (Joppa and Williams 1988), and the recombinant substitution line Langdon#65 (LDN#65), which has a 30-cM region of chromosome 6B from *Triticum turgidum* var. *dicoccoides* (Joppa et al. 1997). This recombinant substitution line was used to construct the BAC library of tetraploid wheat containing 516 096 clones individually maintained on 1344 384-well plates (Cenci et al. 2003).

DNA extraction

Total DNA was extracted from wheat lines following the method proposed by Dellaporta et al. (1983). BAC DNA was extracted from bulked cultures of all clones from each 384-well plate using an AutoGen 850α robot (AutoGen, Framingham, Mass.). The 1344 DNA bulks were organised in 56 superpools including 24 plates each, which were further arrayed in 6 rows × 4 columns to reduce the number of PCRs required to screen the BAC library (Fig. 1). Individual clone identification from the positive plates was performed by colony PCR using a 24 × 16 pooling strategy.

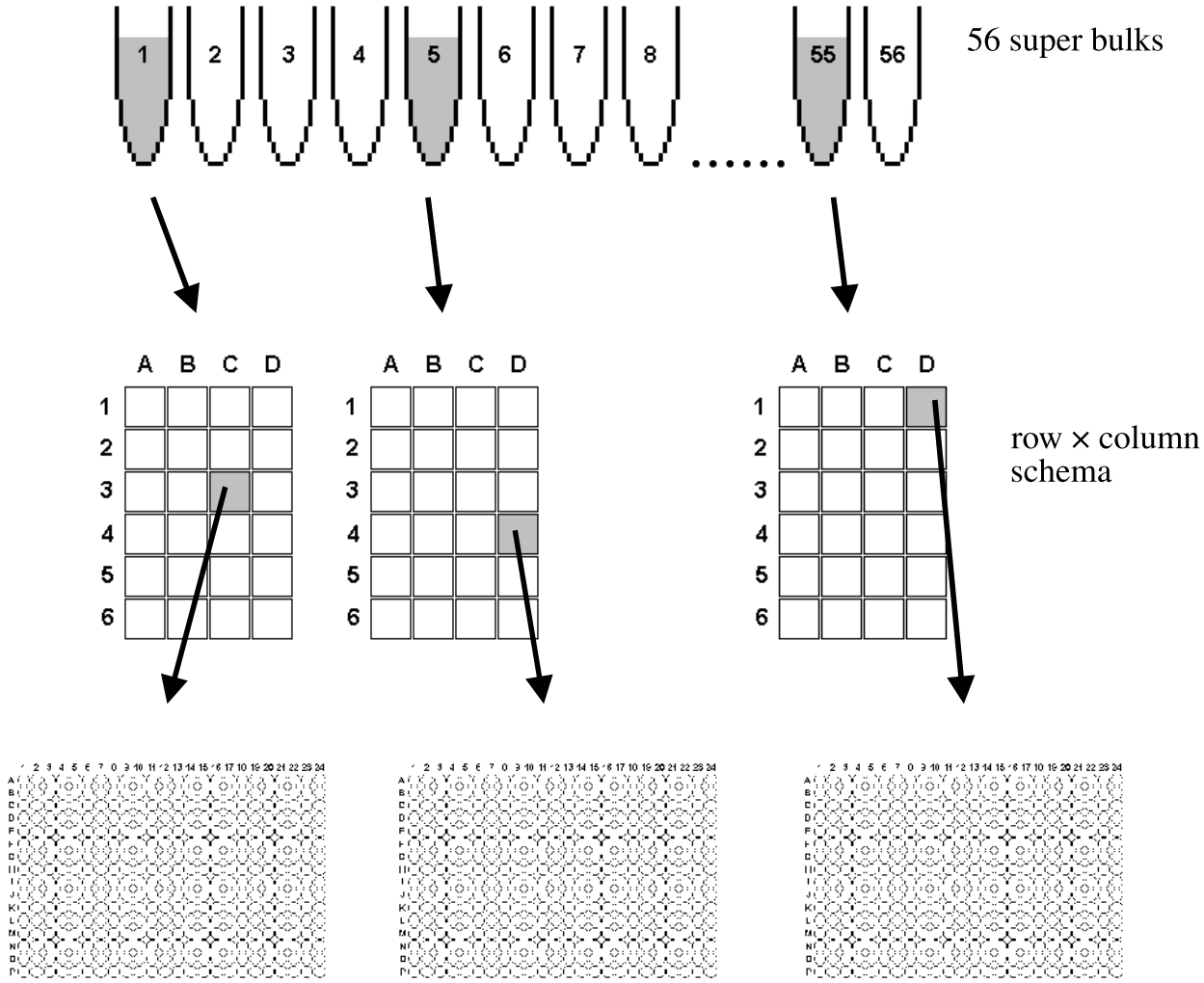
PCR primer design

The National Center of Biotechnology Information (NCBI) and European Bioinformatic Institute (EBI) databases were examined to see if they contained *PSY*, *PDS*, and *ZDS* sequences. Using rice or maize sequences, a BLAST search was carried out to find Triticeae ESTs. PCR primers were designed on the basis of wheat ESTs similar to the target genes using the Primer3 WWW primer tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi; University of Massachusetts Medical School, Worcester, Mass.). At least four primers (two forward and two reverse) were designed for each gene to maximize the probability of amplification success. The *Arabidopsis* and rice sequences were used to predict the exon boundaries within the Triticeae ESTs. Primers were designed avoiding the exon–intron junctions, targeting PCR products in the range of 300 to 600 bp, and generally including at least one intron (Table 1). All primer pairs were tested on genomic DNA of LDN#65 before BAC library screening.

PCR amplification and detection of polymorphisms between A and B genome BACs

PCR amplification consisted of 35 cycles of 1 min at 94 °C, 1 min at 50 °C, and 2 min at 72 °C. PCR amplification products from the selected BACs were separated by electrophoresis (2.0%–2.5% *w*/*v* agarose concentration). If no differences in length were detected between the amplification products from the selected BACs in the agarose gels, conformation-sensitive gel electrophoresis polymorphism (CSGE, Ganguly et al. 1993; Yasukochi 1999) was used to screen for polymorphisms. The PCR product obtained from a positive plate was mixed with the PCR product obtained from all other positive plates. The samples were then denatured for 5 min at 95 °C, rapidly cooled at 4 °C, and loaded on 2.5% *w*/*v* urea-free agarose gel. The presence of additional bands (heteroduplexes) was used as evidence that the two BACs were from different genomes. Finally, PCR prod-

Fig. 1. Schematic representation of PCR screening of the durum wheat BAC library. Each super bulk is a mixture of DNAs from 24 × 384 BAC clones. The positive plates within the super bulks were identified from by a 6 × 4 pooling strategy. The positive clones within the 384-well plates were then identified by colony PCR using a 16 row × 24 column pooling strategy.



ucts from the different BAC clones were screened for polymorphisms using four-cutter restriction enzymes, *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Hin*fI, *Msp*I, *Nde*II, *Rsa*I, and *Taq*I.

Results

Sequences for wheat phytoene synthase (*PSY*), phytoene desaturase (*PDS*), and ζ-carotene desaturase (*ZDS*) and primer design

The BLASTN search using rice mRNA *PSY* sequence (AY024350) resulted in two significant wheat ESTs (BM137086 and BE604139, $E < 1.00 \times 10^{-100}$). Both sequences showed a high similarity in the region corresponding to rice exons 3, 4, and 5 (AY024351). Two pairs of primers were designed based on the wheat BE604139 sequence (Table 1).

The BLASTN search using rice mRNA *PDS* sequence (AF049356) resulted in several Triticeae ESTs showing high similarity to the rice gene ($E < 1.00 \times 10^{-100}$). Many of these ESTs were grouped in Unigene cluster Ta.1201 (http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Ta&CID=1201). Five primers (Table 1) were designed based on the BG908924 and BG908925 wheat sequences corresponding to the 5′ and 3′ ends of cDNA clone TaLr1174B11, and on the BF267352 barley EST.

The BLAST search using maize mRNA *ZDS* sequence (AF047490) resulted in two significant matches with *Triticum aestivum* ESTs, BG909132 and BG909133 ($E < 1.00 \times 10^{-30}$), corresponding to the 5′ and 3′ ends of cDNA clone TaLr1174C11. Two pairs of primers were designed for both sequences (Table 1).

PCR screening of the durum wheat BAC library for *PSY*, *PDS*, and *ZDS*

PSY

PCR amplification of LDN#65 genomic DNA with primer

Table 1. Primers designed for the phytoene synthase (*PSY*), phytoene desaturase (*PDS*) and ζ-carotene desaturase (*ZDS*) genes for the BAC library screening.

Primer name	Sequence (5′→3′)	Source	Position
PSY-F1	TGGGAGAAGAGATTGGAGGATC	BE604139	Exon 3
PSY-F2	GAGACATGATTGAAGGGATGAG	BE604139	Exon 4
PSY-R1	AGTGCCAGCGACGTAGTAGCAG	BE604139	Exon 4
PSY-R2	CTCGGAGGATGTTTGTGAGCTG	BE604139	Exon 4
PDS-F1	TGGCATTCTTGGATGGTAATCC	BG908924	Exon 8
PDS-F2	GGGACTCAAATAACTGGAGATGC	BG908924	Exon 8
PDS-R1	CTTGTTGAAAAGAAGGTGGT	BF267352	Exon 10
PDS-R2	GACCAACTCCAGCATCGAACGG	BG908925	Exon 12
PDS-R3	TTGCTTTACTCTGGTCAGCAGC	BG908925	Exon 12
ZDS-F1	GGCTGCATGTCTTCTTTGGT	BG909132	Exon 3
ZDS-F2	ATAAAGGGGGCATTGTTGGT	BG909132	Exon 4
ZDS-F3	ACAGCATGGAAGGAGCAACT	BG909133	Exon 13
ZDS-F4	AGCCATTCGAAAGAAGCTCA	BG909133	Exon 13
ZDS-R1	GCTCGAACAACTGGGCTTAG	BG909132	Exon 7
ZDS-R2	ATCAGGTGAGCCCTTTAGCA	BG909132	Exon 8
ZDS-R3	CGAATGACGGGCTAAGGTT	BG909133	Exon 13
ZDS-R4	TGTGGGTATCTTCCCAGACA	BG909133	Exon 13

Note: Source, GenBank accession No.; position, the exon position on the rice (PSY and PDS) or *Arabidopsis* (ZDS) genes.

combination PSY-F1–PSY-R1 produced three discrete fragments ranging between 300 and 360 bp (Fig. 2). These sizes were larger than the 200 bp predicted from the cDNA sequence, indicating the presence of at least one intron. This was the expected result since primers PSY-F1 and PSY-R1 correspond to sequences located in exons 3 and 4 in rice *PSY* (AY024351, Table 1). Eight positive plates (31, 40, 70, 527, 529, 608, 998, and 1031) were identified in the PCR screening of the complete BAC library. The amplification products from plates 31, 70, 529, 608, and 1031 showed a higher molecular weight than the amplification products from plates 40, 527, and 998 (Fig. 2). Only one plate from each group was used for the identification of a positive BAC clone from each genome: 527J1 and 529P2. The presence of *PSY* in these BAC clones was confirmed by PCR using the four possible combinations of the four *PSY* primers (Table 1).

PDS

PCR amplification of LDN#65 genomic DNA with primer combination PDS-F2–PDS-R1 produced a single 380-bp fragment. This size was larger than the 200 bp expected from the cDNA sequence, indicating the presence of at least one intron on the wheat genomic DNA. This was expected because these primers correspond to sequences from exons 8 and 10, on the rice genomic sequence (AC079633, Table 1). Six positive plates (118, 252, 405, 433, 970, 1211) were identified in the screening of the BAC library with these primers. Since no difference in length was detected in the agarose gel electrophoresis between the PCR products obtained from the six plates, a screen for polymorphism was performed using conformation-sensitive gel electrophoresis (CSGE) (Fig. 3*a* and 3*b*, data not shown for plate 1211). The observed polymorphism separated the positive clones on plates 118, 252, and 1211 from those on plates 405, 433, and 970. The identification of individual positive BAC clones was performed only for plates 118 and 970 (118P17 and 970M4). The presence of the *PDS* within these BAC clones was confirmed by PCR using the six possible combinations of the four *PDS* primers.

ZDS

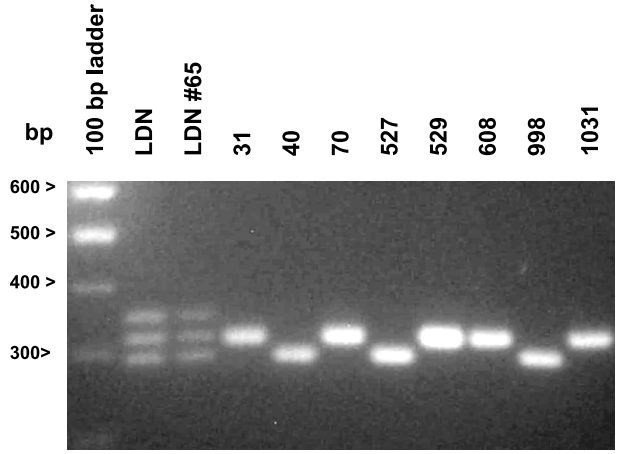
The primers ZDS-F3 and ZDS-R4 were located within the same exon of the *ZDS* and the PCR amplification from genomic DNA of LDN#65 produced the 460-bp fragment expected from the cDNA sequence. These primers were used to screen the BAC library and nine positive plates were identified: 139, 467, 584, 680, 785, 791, 859, 862, and 1307. No polymorphisms were detected among the PCR products from these BAC clones by normal or CSGE electrophoresis. Digestion of the PCR products obtained with the different ZDS primer combinations (Table 1) with different restriction enzymes revealed a *Taq*I polymorphism among the amplification products from primers ZDS-F2 and ZDS-R2. BAC clones 139, 467, 862, and 1307 were assigned to one group and BAC clones 584, 680, 785, 791, and 859 to the other one (Fig. 4). The final BAC clone identification was performed only for plates 467 and 791 (467K1 and 791A16). PCR products from primers corresponding to different exons in the *Arabidopsis ZDS* (AF121947) were much larger than expected from the cDNA sequence confirming the presence of introns in similar positions in the wheat and *Arabidopsis* genes (Table 1).

Chromosomal mapping of wheat *PSY, PDS,* and *ZDS*

PSY

Amplification of LDN#65 genomic DNA with primers PSY-F1–PSY-R1 produced fragments of three different lengths. Two of these fragments were assigned to BAC clones 527J1 and 529P2. The smallest PCR product, similar to the one obtained from BAC clone 527J1, was mapped on chromosome 5A (band absent in substitution line LDN5D(5A), Fig. 5). The fragment with intermediate mobility, similar to

Fig. 2. PCR amplification of genomic DNA from *Triticum turgidum* var. *durum* 'Langdon' (LDN), LDN#65, and DNA mixes from 8 positive 384-well plates of the LDN#65 BAC library (primers PSY-F1 and PSY-R1).



the one obtained from BAC clone 529P2, was mapped on chromosome 5B (band absent in LDN5D(5B), Fig. 5). The highest band was absent in the amplification products from LDN5D(5A) and LDN5D(5B) substitution lines, suggesting that it was the result of a 5A–5B heteroduplex molecule. The PCR pattern obtained with a mixture of DNA from BAC clones 527J1 and 529P2 showed the same band and confirmed this hypothesis (data not shown). Substitution lines LDN5D(5A) and LDN5D(5B) showed an additional PCR product, assigned to the 5D chromosome. Additional bands on LDN5D(5A) and LDN5D(5B), with a similar mobility to the 5A/5B heteroduplex, are probably 5A/5D and 5B/5D heteroduplex molecules (Fig. 5).

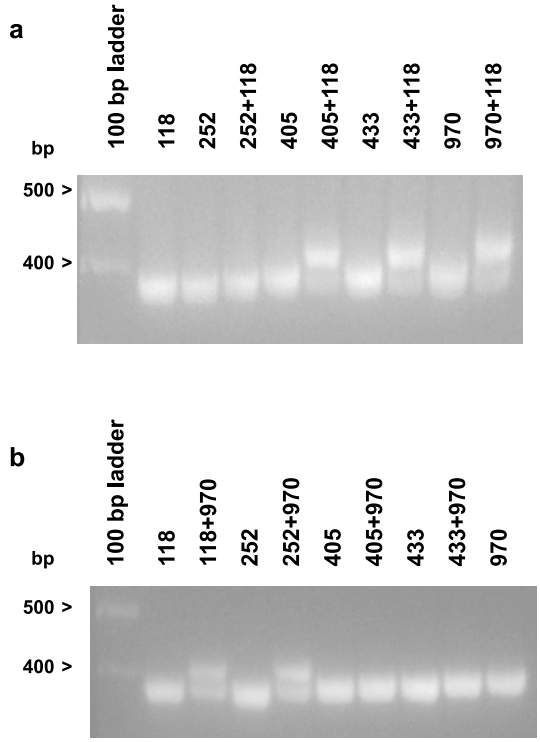
PDS

A single PCR fragment was amplified from LDN#65 genomic DNA using all *PDS* primer combinations. CSGE polymorphism between BAC clones showed that this band corresponded to at least two different products having very similar, if not identical, size. To facilitate the mapping of this gene, PCR products (PDS-F2/PDS-R3) from BAC clones 118P17 and 970M4 were screened for polymorphisms with different restriction enzymes. A *Taq*I polymorphism was found and used to assign clones 970M4 and 118P7 to chromosomes 4A and 4B, respectively. An additional PCR fragment was detected on both LDN4D(4A) and LDN4D(4B) substitution line patterns indicating the presence of a copy of the *PDS* on chromosome 4D (data not shown).

ZDS

The *Taq*I polymorphism found between the A and B genome BACs selected with the *ZDS* was used to assign BAC 791A16 to chromosome 2A and BAC 467K1 to chromosome 2B. An additional band was present on both LDN2D(2A) and LDN2D(2B) substitution line patterns, which indicates the presence of a copy of the *ZDS* on chromosome 2D (data not shown).

Fig. 3. Conformation-sensitive gel electrophoresis (CSGE) of PCR products obtained with PDS-F2–PDS-R1 primer combination on positive 384-well plates from the LDN#65 BAC library. The "+" sign indicates lanes corresponding to mixtures of PCR products from two plates. Arrow indicates the heteroduplex band.



Discussion

PCR screening of the BAC library

Triticeae genomes are very large (Arumuganathan and Earle 1991) and many clones are needed to obtain representative BAC libraries (Moullet et al. 1999; Lijavetzky et al. 1999; Cenci et al. 2003). Therefore, a multi-step approach (Green and Olson 1990) was used to screen the 516 000 clones from the LDN#65 BAC library by PCR. The pooling strategy used in this study required 56 PCRs for the superpools (9216 BAC clones each) and 50 additional PCRs for each positive superpool. Alternative pooling strategies identifying positive plates by two or more coordinates, like multidimensional screening, are also available (O'Sullivan et al. 2001).

The number of positive BAC clones found with the primers for the *PSY*, *PDS*, and *ZDS* genes (23) was slightly lower than the 30 positives expected from the 5X coverage for each genome. Only one positive BAC clone was found on each of the six positive plates analysed in detail to identify individual positive BACs. However, it is possible that some of the other 17 positive plates could have included more than one positive BAC clone. The probability of finding two or more positive BACs within a single 384-well plate in this library (5× coverage) is very low (0.38%); therefore, the probability of finding one or more positive BAC clones within the 17 plates not analysed for individual positive BACs is relatively small (6.5%). Consequently, it is unlikely that this

Fig. 4. *Taq*I restriction pattern of PCR products from positive 384-well plates from the LDN#65 BAC library (ZDS-F2–ZDS-R2 primer combination).

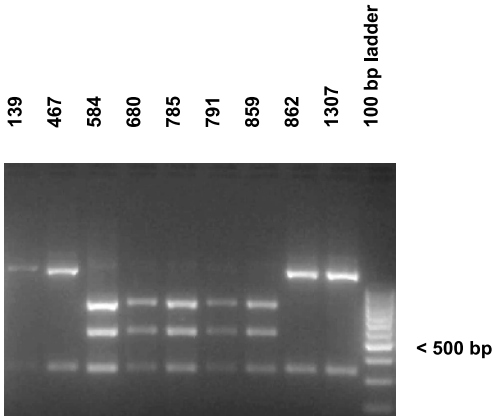
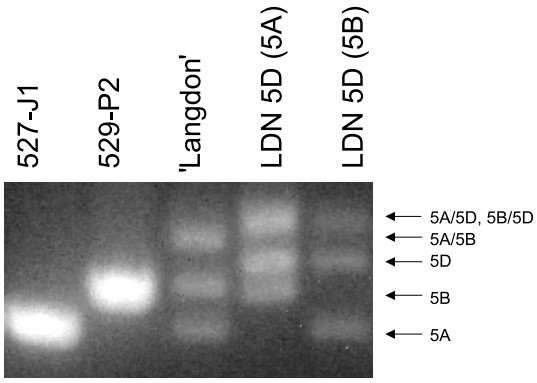


Fig. 5. Chromosomal location of 'Langdon' PCR products obtained with PSY-F1–PSY-R1 primer combination. Heteroduplex artefacts are indicated by arrowheads.



was a major cause of the lower than expected number of positive clones. Additional causes could include the loss of BAC clones from slow growing colonies during the growing of the initial pools of 384 BACs, or simply random sampling variation (P = 0.115, based on a cumulative Poisson distribution).

Mapping of BAC clones in tetraploid wheat

Approximately 900 000 Triticeae ESTs are now available in the DNA sequence databases (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html, accessed December 2003). Therefore, there is now a good probability of finding wheat or barley ESTs based on the sequence information from similar genes from other species. Once a Triticeae EST is identified, it is possible to predict the exon boundaries using the annotation of the corresponding rice or *Arabidopsis* genes. The exon–intron structure is generally well conserved between wheat and rice (Dubcovsky et al. 2001), thus facilitating the design of primers flanking intron regions. All the intron positions predicted for these three genes based on the rice and *Arabidopsis* annotations were validated by the PCR amplifications from wheat genomic DNA.

The inclusion of introns in the amplified PCR products increases the probability of detecting polymorphisms between sequences from the different wheat genomes. Owing to their ability to accumulate mutations without modifying gene function, introns are known to evolve more rapidly than exons.

Length polymorphisms were the easiest to detect and to use to distinguish clones from the different genomes, followed by conformation-sensitive gel electrophoresis (Ganguly et al. 1993). This last method is similar to that proposed by Yasukochi (1999), but run on a common non-denaturing agarose gel. Heteroduplex bands can complicate the analysis in polyploid species, and strategies to eliminate them have been proposed (Thompson et al. 2002). In the present paper, the heteroduplex formation is exploited to distinguish PCR products amplified from different genomes. The detection of useful heteroduplex bands is favoured by the presence of introns and the reduced size of the amplification products. Small mobility differences cannot be resolved on large heteroduplex molecules on 2.5% *w*/*v* agarose gel.

Genes for enzymes of the carotenoid biosynthesis pathway

Genes encoding PSY, PDS, and ZDS were located on chromosome groups 5, 4, and 2, respectively. *PDS* chromosomal location is consistent with recent data of EST deletion mapping: one of the EST clones grouped on Unigene cluster Ta.1201 (WHE1655–1658_O12_O12) was mapped on BINS 4AS4-0.63-0.76, 4BL5-0.86-1.00, and 4DL13-0.56-1.00* by hybridization with a panel of deletion lines (wEST-SQL, http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi). *PSY* and *ZDS* localization has not yet been reported.

Interestingly, *PSY* and *ZDS* were mapped on chromosome groups 2 and 5, where QTLs for semolina and pasta colour have been detected (A. Blanco, unpublished data). Since pasta and semolina colour are determined in part by carotenoid concentration, enzymes from the carotenoid biosynthesis pathway are good candidate genes to explain these differences.

Sequences of the A and B genome copies of these genes can be obtained from the BACs selected in this study and used to design genome-specific primers. These primers can then be used to find sequence polymorphisms between the parental lines of the current linkage maps of tetraploid wheat, and to determine their exact map position. This information will be essential to determine if there is an association between the QTLs for semolina and pasta colour on chromosomes 2 and 5 and the genes from the carotenoid biosynthesis pathway.

In conclusion, the strategy used here to isolate BAC clones containing genes unknown in durum wheat but sequenced in other cereals was successful and can be extended to other genes previously identified in model plant species. This study also presents several methods to distinguish PCR products amplified from the different genomes in a BAC library from a polyploid species. Finally, this study provided the chromosome location of the central genes for the important carotenoid biosynthesis pathway.

Acknowledgements

The research was supported by Ministero delle Politiche Agricole e Forestali (MiPAF), Italy, Project "FRUMISIS", and by Ministero della Ricerca Scientifica e Tecnologica,

Project “CEGBA”. J.D. acknowledges financial support from USDA–IFAFS competitive grant No. 2001-04462.

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