

# CELL BIOLOGY & MOLECULAR GENETICS

## Identification of Expressed Sequence Markers for a Major Gene-Rich Region of Wheat Chromosome Group 1 Using RNA Fingerprinting–Differential Display

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### ABSTRACT

This study demonstrates a successful application of RNA fingerprinting–differential display technique in identifying expressed sequence markers for a small targeted region of the wheat (*Triticum aestivum* L.) genome. Wheat genes are present in clusters spanning about 10% of the genome. One of the important gene-rich regions is present on the short arm of wheat homoeologous group 1 chromosomes around fraction length 0.8 ('1S0.8 region'). The region is about 0.1% of the wheat genome and is flanked by the breakpoints of deletion lines 1BS-4 and 1BS-19. The objective of this study was to identify expressed sequence markers for the region. First-strand cDNA of poly A<sup>+</sup> mRNA pooled from various developmental stages of the two deletion lines were PCR amplified in the presence of <sup>32</sup>S by means of 90 pair-wise combinations of 19 primers. Amplification products were size-separated on a denaturing polyacrylamide urea gel. A total of 6840 fragment bands were amplified, of which 65 were present in the deletion line 1BS-4, but missing in 1BS-19. These 65 fragment bands were cut out of the gel, reamplified, and used as probes for gel-blot DNA analysis of group 1 nullisomic-tetrasomic lines and the two deletion lines. Nineteen of the 65 fragment bands detected a smear pattern and thus were not mapped. Of the remaining 46 probes, 22 mapped to wheat homoeologous group 1 and seven mapped to the '1S0.8 region'. The same approach can be used to target other wheat gene-rich regions bracketed by deletion breakpoints.

WHEAT GENES ARE PRESENT in clusters encompassing small chromosomal regions (Faris et al., 2000; Gill et al., 1996a,b; Sandhu et al., 2001). Genes on wheat homoeologous group 1 chromosomes are present in eight clusters, physically spanning ~10% of the chromosomal region (Gill et al., 1996a,b; Sandhu, 2000). Among these, the gene-rich region present at fraction length (FL) 0.8 of the short arm ('1S0.8 region') is very important. By comparative mapping, 75 agronomically important genes were localized to the 1S0.8 homoeologous region among various Triticeae species (Sandhu et al., 2001). These include six leaf rust (*Lr*), five yellow rust (*Yr*), four stem rust (*Sr*), four mildew (*Mla*), one barley rust (*Pa*), and two powdery mildew (*Pm*) resistance genes. The region also contains genes for grain quality proteins such as gliadin (*Gli*), glutenin (*Glu*), triticin (*Tri*), and hordein (*Hor*), resistance to preharvest sprout-

ing (*Qphs.cnl*), restorer for cytoplasmic male sterility (*Vi* and *Rf*), and a tiller-inhibitor gene (*Tin*) (Sandhu et al., 2001). This gene-rich region is best localized on chromosome 1B, where it maps to the mid-satellite region of the chromosome and is flanked by the breakpoints of two single-break deletion lines, 1BS-4 and 1BS-19 (Gill et al., 1996a) (Fig. 1). Both deletion lines are in the background of cultivar Chinese Spring (CS) and differ only in the size of the terminal deletion, which is smaller in 1BS-4 compared to that in 1BS-19 (Endo and Gill, 1996). On the basis of cytological measurements, the size of the region was estimated to be 15 megabases (Mb) or about 0.1% of the wheat genome (Sandhu et al., 2001).

High density genetic linkage maps are available for wheat and its relatives such as *T. monococcum* L., *Aegilops tauschii* Coss., barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), etc. There are about 4000 markers present on one or more of the Triticeae maps (Grain-Genes, <http://wheat.pw.usda.gov/>; verified February 14, 2002). Comparative mapping can make use of the mapping information across species because gene synteny is highly conserved among the species of Triticeae and moderately conserved among various tribes of the family Poaceae (Bennetzen et al., 1998; Devos and Gale, 2000; Hart, 1987). A combination of comparative mapping and deletion line-based physical mapping was used to identify 44 markers for the '1S0.8 region' (Sandhu et al., 2001). With an average size of BAC clones to be 100 kilobases (kb), one marker can span up to 150 kb of BAC clones. Forty-four markers, therefore, can span up to 6.6 Mb region. Estimated size of the '1S0.8 region', however, is about 15 Mb (Sandhu et al., 2001). Therefore, additional markers are needed to construct a contiguous map for the whole region. Furthermore, despite hexaploid nature of wheat genome, a major fraction of wheat genes show monogenic inheritance (McIntosh et al., 1998; Sandhu, 2000). Therefore, identification of expression markers specific to one of the wheat genomes is of great value.

RNA fingerprinting–differential display (RNAF–DD) is a powerful technique to identify and isolate genes that are differentially expressed between two RNA populations (Liang and Pardee, 1992). A key element of this technique is to use a set of oligonucleotide primers, one being anchored to the polyadenylated tail of a subset of mRNAs and the other being short and arbitrary in sequence so that it anneals at a different position relative to the first primer. RNA fingerprinting by arbitrarily primed PCR and its variants have been used

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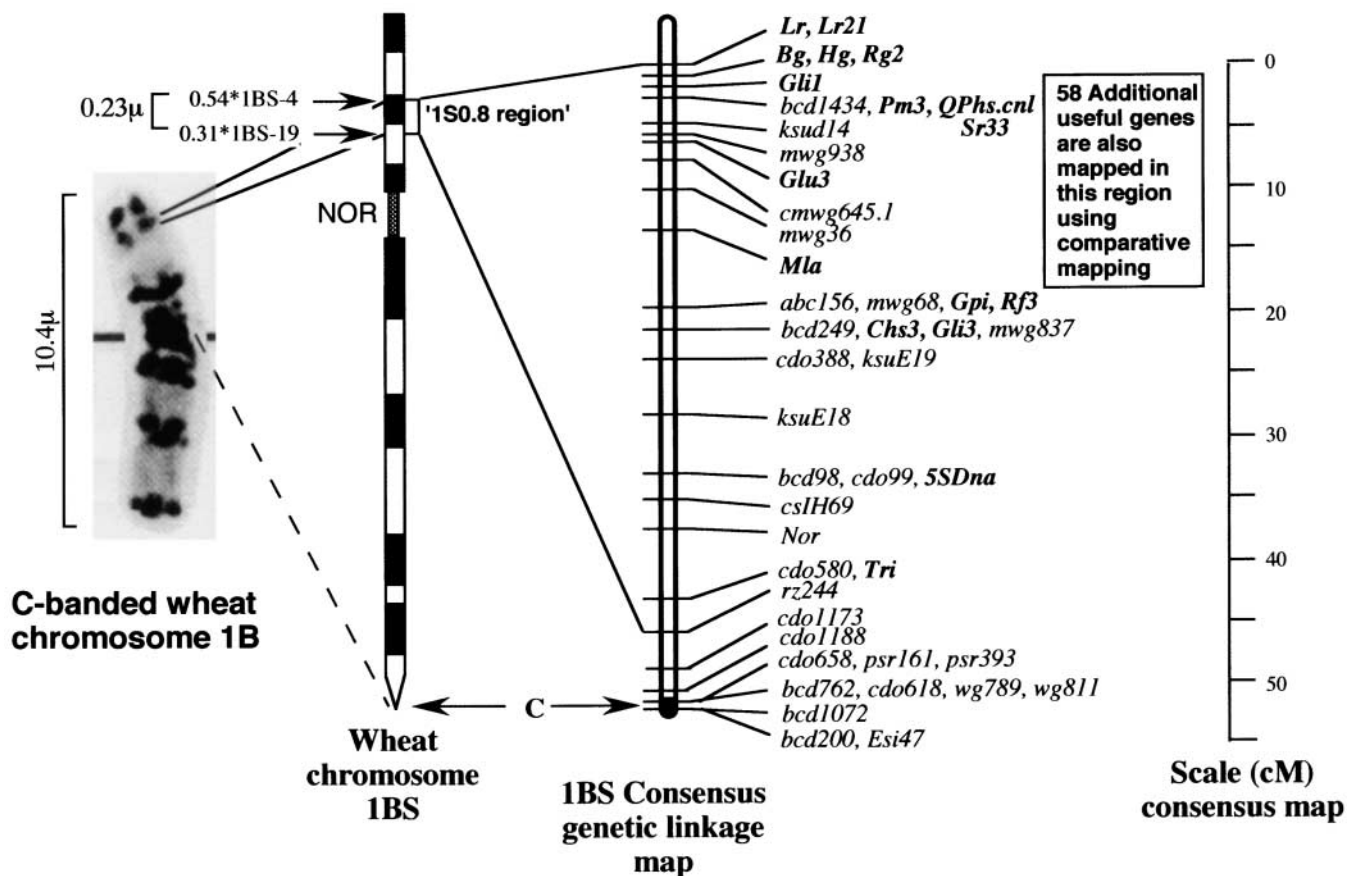


Fig. 1. C-banding pattern (left) (Gill et al., 1991) and ideogram (center) of chromosome 1BS of wheat in comparison with the consensus genetic map (right) (Van Deynze et al., 1995). Asterisk represents fraction length of the satellite. Black boxes represent C-bands on the chromosome. NOR, nucleolar organizer region.

successfully to isolate differentially expressed genes in a large number of experimental systems (Diachenko et al., 1996; Liang et al., 1993). In wheat, RNAF-DD has been used for the isolation of early salt responding genes, cDNA expressed in spikelets, and identification of different members of heat shock protein families (Joshi and Nguyen, 1996; Joshi et al., 1996, 1997; Nemoto et al., 1999; Ogihara et al., 1998). A major complication in this procedure is the identification of real positives that are normally differentiated from false positives by Northern blot hybridization analysis or by quantitative RT-PCR (Liang and Pardee, 1992). If the transcript of the gene of interest is of low-abundance, Northern analysis may not be sensitive enough to identify the real positives. Furthermore, Northern blot hybridization or RT-PCR based methods will not reveal the location of a differentially expressed gene in the genome. For the experiments where main objective is to target a particular gene, physical location of a potential positive clone is of tremendous value.

Availability of unique aneuploid stocks (Endo and Gill, 1996) makes wheat an ideal system in which to use the RNAF-DD technique for marker enrichment and gene cloning. The wheat nullisomic-tetrasomic lines (where a pair of chromosomes is missing, the loss of which is compensated for by the double dose of one of its two homoeologous chromosomes) and the wheat ditelosomic lines (missing a pair of chromosome arms

(Sears, 1954) are invaluable for interchromosomal arm mapping of any single (or a few) copy DNA or RNA fragments. Further, there are 436 single-break deletion lines involving all wheat chromosomes that can be used to target any region in the wheat genome and can reveal the precise physical location of any fragment. Essentially, any fragment can be mapped because this mapping procedure is less dependent on the probe's ability to detect polymorphism.

The objective of this study was to identify expressed gene markers in the '1S0.8 region' of wheat using RNAF-DD.

**MATERIALS AND METHODS**

**Plant Material**

Two single-break deletion lines 1BS-4 and 1BS-19, flanking the '1S0.8 region' with their breakpoints, were used for RNAF-DD. Wheat homoeologous group 1 nullisomic-tetrasomic lines along with the two deletion lines were used for DNA gel-blot analysis to confirm the location of putative positive fragments. Plant materials were obtained from the wheat genetic resource center (WGRC), Manhattan, KS.

**Poly(A)<sup>+</sup> RNA Isolation**

The deletion lines 1BS-4 and 1BS-19 were grown under similar conditions in a greenhouse. Equal quantity of plant tissue at seedling, tillering, booting, and at ear emergence

stages was pooled. Total RNA from each deletion line was isolated from the pooled tissue by means of the guanidinium thiocyanate–cesium chloride method (Sambrook et al., 1989) with few modifications. The tissue was ground to a fine powder in liquid nitrogen and was suspended in a guanidinium thiocyanate buffer containing 1% (v/v) mercaptoethanol and 0.5% (w/v) sodium lauryl sarcosinate. Samples were centrifuged at 6000 g for 15 min. The supernatant was layered on a 5.7 M CsCl –0.01 M EDTA (ethylenedinitrilo tetraacetic acid disodium salt) solution and centrifuged in a swinging bucket rotor at 215 000 g for 16 h. The RNA pellet was washed with 70% (v/v) ethanol and resuspended in TE buffer containing 0.1% SDS. PolyA<sup>+</sup> RNA was isolated following a standard protocol (Sambrook et al., 1989).

### RNA Fingerprinting–Differential Display (RNAF–DD)

RNAF–DD reactions were carried out with the Delta RNA fingerprinting kit (Clontech Lab, Inc., Palo Alto, CA), following recommended protocols. First-strand cDNA was synthesized from poly A<sup>+</sup> RNA of the deletions 1BS-4 and 1BS-19 by means of Moloney murine leukemia virus (MoMLV) reverse transcriptase. About 20 ng of the first strand cDNA was PCR amplified by means of a ‘P’ and a ‘T’ primer in the presence of <sup>35</sup>S dATP. The ‘T’ primers have a common 19-bp sequence at the 5′ end followed by nine thymidine bases. The two bases at the 3′ end are variable and are present in all possible pair-wise combinations of A, C, and G. The ‘P’ primers share a common sequence of 16 bases at the 5′ end but contain a variable sequence of nine or 10 bases at the 3′ end. The variable sequences of the ‘P’ primers were selected on the basis of some commonly occurring sequence motifs in the coding regions of eukaryotic mRNAs. All 90 possible primer combinations involving 10 ‘P’ and nine ‘T’ primers, were used in the study (Clontech Lab, Inc.).

The PCR conditions for RNAF–DD reactions were as follows: one cycle of 5 min at 94°C, 5 min at 40°C, 5 min at 68°C; 2 cycles of 2 min at 94°C, 5 min at 40°C, 5 min at 68°C; 25 cycles of 2 min at 94°C, 1 min at 60°C, 2 min at 68°C, followed by 7 min at 68°C. The amplification products were size separated on a 0.4-mm-thick denaturing 5% (w/v) polyacrylamide, 8 M urea gel following standard sequencing gel protocols (Sambrook et al., 1989). Only one-fifth of the amplification product was loaded on the gel. The gels were pre-run at 33 mA constant current for 30 min and run at 80 W constant power for 3 to 4 h. The gels were then soaked in TE buffer for 5 min and blotted onto Whatman 3MM sheets. The gel side was covered with plastic wrap and dried for 2 h at 80°C on a gel drier (BIORAD, Richmond, CA). Upon drying, the plastic wrap was removed and an X-ray film was placed on the dried gel and exposed for 3 to 7 d. The fragment bands present in 1BS-4 but absent in 1BS-19 were identified and cut out of the gel. The DNA was eluted by boiling the gel slice in 50 mL of sterile water for 10 min. Eluted DNA was PCR reamplified by means of the same primer set that was used for the RNAF–DD reaction.

### Confirmation of the Positive Clones

To identify expressed sequences specific for the ‘1S0.8 region’, the putative positive fragment bands were used as probes for gel-blot DNA hybridization of blots containing group 1 nullisomic-tetrasomic lines and the two deletion lines, 1BS-4 and 1BS-19. Genomic DNA was digested with either *Eco*RI or *Hind*III restriction enzymes. Gel-blot DNA hybridization procedures were as previously described (Gill et al., 1993).

### DNA Cloning

The T-A cloning kit (Promega, Madison, WI) was used to clone all seven fragments mapping in the ‘1S0.8 region’. Inserts from white colonies were used as probes on the blots described above to identify fragments mapping in the target region.

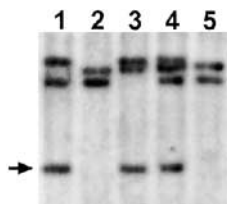
### RESULTS

RNAF–DD reactions were performed on cDNA of deletion lines 1BS-4 and 1BS-19 by means of 90 primer combinations to identify cDNAs differentially expressed from the B genome homoeologue of the ‘1S0.8 region’. Amplification products from 1BS-4 and 1BS-19 were run side by side on denaturing polyacrylamide urea gels (Fig. 2). A total of 6840 fragment bands were amplified by means of 90 primer combinations. There were an average of 76 bands per primer combination. Except for few bands, the fragment band pattern in both deletion lines was almost identical. A total of 65 fragment bands was present in deletion line 1BS-4 and



Fig. 2. Autoradiogram of an RNAF–DD gel. Lanes 1 and 2 represent RNAF patterns of deletion 1BS-4 and 1BS-19, respectively. One of the fragment bands present in 1BS-4 but missing in 1BS-19 is marked by an arrow. One of the fragment bands present in 1BS-19 but missing in 1BS-4 is marked by a bullet.





**Fig. 3.** Gel-blot DNA hybridization of a positive clone (UNL12) on genomic DNA of group 1 nullisomic-tetrasomic lines, 1BS-4 and 1BS-19. Lane 1, Nulli 1A-Tetra 1B; Lane 2, Nulli 1B-Tetra 1D; Lane 3, Nulli 1D-Tetra 1A; Lane 4, 1BS-4; Lane 5, 1BS-19. Arrow represents B fragment band present in 1BS-4 and missing in 1BS-19.

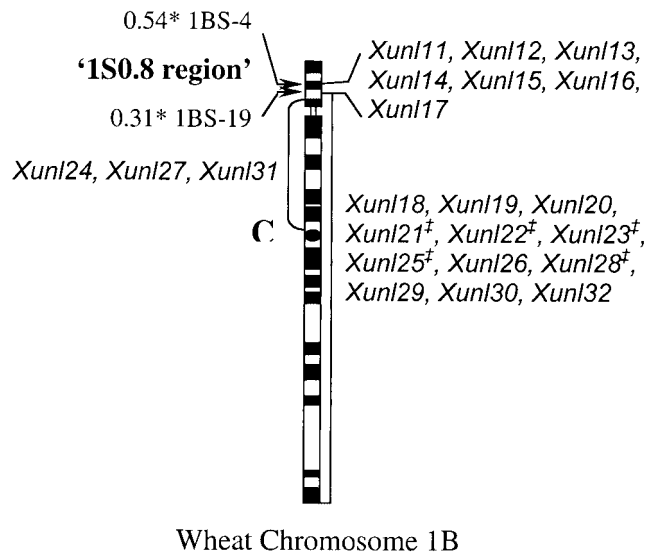
missing in 1BS-19 (see arrow, Fig. 2). These potential positives also included fragment bands differing in relative intensity. We were inclusive as the deletion line-based confirmation of positives is easy and accurate. Unexpectedly, another 44 fragment bands were present in 1BS-19 but absent in 1BS-4 (see bullet mark, Fig. 2). These fragment bands may suggest secondary deletions in 1BS-4 line (Sandhu, 2000). The confirmation of secondary deletions is very difficult and time consuming, therefore, these fragment bands were not characterized further.

Reamplified DNAs from the 65 fragments were used as probes for gel-blot DNA hybridization analysis of group 1 nullisomic-tetrasomic lines, 1BS-4, and 1BS-19 to identify fragments mapping in the '1S0.8 region'. Nineteen of the 65 potential positives were not amenable to mapping because multiple fragment bands and/or smear patterns were detected during gel-blot analysis. Of the remaining 46 probes, 22 mapped to wheat homologous group 1, as these detected missing fragment band(s) in one or more of the group 1 nullisomic-tetrasomic lines. Seven (*Xunl11*, *Xunl12*, *Xunl13*, *Xunl14*, *Xunl15*, *Xunl16*, and *Xunl17*) of these 22 probes mapped in the '1S0.8 region', as the corresponding 1BS specific fragment bands were present in deletion 1BS-4 but absent in 1BS-19 (Fig. 3). For five (*Xunl21*, *Xunl22*, *Xunl23*, *Xunl25*, and *Xunl28*) of the remaining 15 group 1 probes, B genome-specific fragments were not resolved by the two restriction enzymes used. The remaining 10 probes, however, mapped to other chromosomal regions of group 1 (Fig. 4).

Because gel-purified fragments may be a mixture of more than one type of same sized DNA, the seven '1S0.8 region' probe fragments were cloned with the T-A cloning system (Promega). Three to four cloned inserts, representing each fragment band, were again tested by DNA gel-blot analysis, as mentioned earlier. Three out of the seven '1S0.8 region' fragments contained two different types of cloned inserts, indicated by two distinct restriction patterns. The other four fragments contained one type of fragment, as all cloned inserts representing a fragment band detected only one restriction pattern. The cloned fragments showed better resolution and cleaner banding patterns on DNA gel-blot analysis as compared with the PCR amplified mixed fragments.

## DISCUSSION

This study demonstrates a successful application of RNAF-DD technique in identifying expressed se-



**Fig. 4.** Physical mapping of group 1 probes identified by RNAF-DD, on chromosome 1B. Asterisk represents fraction length of the satellite. ‡ represent markers for which B-genome band was not resolved. Black boxes represent C-bands on the chromosome.

quence markers for a small targeted region of the wheat genome. Two single break deletion lines (1BS-4 and 1BS-19) used in the study flank an important gene-rich region of the wheat genome. This gene-rich region is present in the middle 23% (between FL0.54 and FL0.31) of the satellite on chromosome 1B (Endo and Gill, 1996; Gill et al., 1996a). Cytological estimations suggest that 1BS satellite is about 1  $\mu$  in length, which roughly translates to about 68 Mb of DNA (Gill et al., 1991). The estimated size of the '1S0.8 region' is, therefore, about 15 Mb. The haploid wheat chromosome complement is about 16 000 Mb of DNA and the size of the gene-rich region is approximately 0.1% of the total wheat genome. Assuming a uniform distribution of genes in the genome, on average one out of every 1000 random probes should map in this region. Since most wheat markers and genes are confined to about 10% of the genome encompassed by the gene-rich regions such as '1S0.8 region' (Faris et al., 2000; Gill et al., 1996a,b; Sandhu, 2000; Sandhu et al., 2001), one out of 100 random markers is expected to map in the '1S0.8 region'. The observed number of random probes mapping in the '1S0.8 region' was nine out of a total of 546 clones in another experiment (Boyko et al., 1999). In the present experiment, however, seven out of 46 probes mapped in the '1S0.8 region'. The present method, therefore, resulted in about 10-fold enrichment of the probes for the target region. Moreover, B fragment bands for five probes mapping to group 1 were not resolved by restriction enzymes used, suggesting that these probes may also be present in the '1S0.8 region'.

All wheat genes are expected to have three orthologues corresponding to the three genomes. Still, a significant number of agronomically important genes seem to express mainly from one genome, therefore, isolating cDNAs following a similar type of expression pattern would be of a great value. On the basis of the segregation pattern, four of the 34 agronomically impor-

tant genes present in the '1S0.8 region' seem to express from all three genomes, one from two genomes, and the remaining 29 express mainly from one of the three genomes (McIntosh et al., 1998; Sandhu, 2000 for review). These numbers probably do not represent segregation of all wheat genes, as genes following simpler inheritance are more likely to be studied first. Still, many useful genes segregate in 3:1 ratio indicating that only one of the three copies is expressed. The seven cDNA markers identified in this study are most likely specific for chromosome 1B expression because both deletion lines used in this study have a Chinese Spring background and differ only for the part of chromosome 1B that is present in 1BS-4 but missing in 1BS-19 (Endo and Gill, 1996). The corresponding region on the two homoeologues (1A and 1D) should, however, be identical between the two deletion lines. The expression markers, therefore, can be used to study dosage affect in wheat, which may help in understanding the differences between expression levels of different copies of a same gene. The seven marker genes will not only be valuable for cloning the useful genes of chromosome 1B, but also are more likely to be wheat specific. Approximately one third of the genes from each organism are expected to have unique function and perhaps are the key to the identity of the organism (Rubin et al., 2000). The genes, which evolved after divergence and polyploidization, probably contribute more to the identity of wheat. Genes different among three wheat genomes are more likely to be wheat specific. Chromosome specific expression of a gene may suggest either that the other two orthologues have become silent or that the gene has acquired a different function during evolution.

Seven of the 46 usable fragments mapped to the '1S0.8 region'. A clone not mapping in the '1S0.8 region' is a false positive, and has equal chance of mapping to any wheat chromosome. Theoretically, 14% (1/7th, corresponding to seven wheat groups) of the random markers should map on group 1. However, 22 of the 46 markers (48%) mapped to group 1, which is significantly higher than the expected number. One possible explanation is that a terminal deletion in a chromosome may change the chromatin structure of the remaining arm, thereby changing its gene expression. Different breaks may affect chromatin structure differently. The effect of chromatin structure on transcriptional regulation of gene expression is well established for many genes (Felsenfeld, 1996; Wolffe, 1994). Small interstitial deletions are known to affect gene expression possibly through changes in chromatin structure. Deletion of one or more core histone genes in *Saccharomyces cerevisiae* leads to constitutive expression of some inducible genes (Grunstein, 1990). Another reason for gene expression differences for structurally identical genes in the two deletion lines may be the relative position of the telomere and the centromere. Both centromeres and telomeres are known to affect gene expression (Ekwall et al., 1999).

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