Physical mapping of a low-copy DNA sequence in rye (Secale cereale L.)

(in situ hybridization/ biotin labeling/ gene mapping)

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ABSTRACT A 900-base-pair (bp) sequence from a cDNA clone of the rye endosperm-storage-protein gene Sec-I was labeled with biotin and hybridized to Secale cereale ‘Blanco’ chromosomes. Hybridization was seen on the satellite part of the short arm of chromosome 1R, where the Sec-I gene has been genetically mapped, in ~8.5% of the cells analyzed. The clone cross-hybridized at a lower frequency to rye chromosome arms 1R (long) (4.4%) and 2R (short) (2.6%), where two additional rye storage-protein loci, Sec-3 and Sec-2, respectively, have been mapped. A fourth hybridization site was observed on the short arm of chromosome 6R at a frequency of 3.0%. The cross-hybridization is attributed to a combination of residual sequence homology between the protein loci and the low-stringency conditions used in in situ hybridization. In situ hybridization mapping, in combination with chromosome walking by using molecular techniques, is suggested as an excellent approach to physical mapping of chromosomes.

In situ hybridization is a powerful tool for establishing the physical location of DNA sequences on chromosomes (1). Henderson (2) showed that the technique can successfully map both highly repetitive and unique sequences in animals. The technique has been improved to the point where sequences as small as 500 base pairs (bp) have been detected in human chromosomes (3). However, the results in plant systems have not been so finely resolved; most data have come from mapping highly repetitive sequences (4–11). With plants it appears that low mitotic indices and the presence of cell-wall material and associated cytoplasmic debris in chromosome preparations hinder hybridization of low-copy-number sequences to the chromosome, hinder their detection, and enhance the nonspecific binding of labeled probes (12). In addition, the cooling and condensation of plant chromosomes in mitosis vary from species to species and between preparations. Thus, the observation of low-copy or unique-sequence probes in plants has been difficult.

Recently, in situ hybridization of low-copy or unique sequences in plants has been reported (12–17). The problems associated with poor visualization of probes on plant-chromosome preparations appear to have been overcome by the use of protoplasts instead of the standard root-tip squashes (12–17) and by the use of pachytene spreads (16). The visualization process has been accomplished by using autoradiography (13–16) and various forms of biotin labeling with enzyme–conjugate reporters (12, 13, 17). Autoradiographic methods have, at least, two distinct disadvantages: (i) They generally require fairly long exposure times for detection, and (ii) the high degree of background labeling can make accurate mapping difficult. The biotin–enzyme system appears to eliminate these disadvantages in that preparations can be hybridized and scanned on the same or following day, and little background hybridization is visible.

As previously mentioned, the use of root-tip squashes severely limits detection and hybridization, whereas the use of protoplasts has the disadvantage of resulting in a very low mitotic index. Both techniques have been used on plant preparations; however, the protoplast technique appears superior (ref. 12; J.P.G. and C.L.M., unpublished data). The efficiency of in situ hybridization depends on the probe having access to the denatured chromosomes, and the protoplast technique appears to facilitate this. The increased frequency with which labeled sequences can be detected when using protoplasts more than offsets the low mitotic index presently obtainable in cereals. The problems associated with using either metaphase or pachytene preparations increase with the number and size of the chromosomes, and good spreads of complete cells are difficult to find. The existing techniques apparently will require modification to accommodate the peculiarities of each cereal species or genus (18).

There have been reports that the location of genes on a recombinational map may be very different from their location on a physical map (19–21). Estimates of the amount of DNA present per centimorgan (cM, unit of recombination measuring genetic distance) vary greatly for the same genome. Furthermore, one cannot assume that the relationship between crossover units and physical distance will be constant in different parts of the same genome. Areas where almost no recombination occurs (cold spots) and contrasting areas of high recombination (hot spots) are known. For example, within the bronze (Bz) locus of maize (Zea mays L.), recombination levels are 100-fold higher than in the adjacent areas between the flanking markers (22). Dooner (23) estimated that there are 14 kilobases (kb) per cM at the Bz locus as compared with ~3000 kb per cM for the entire genome. Finally, inversions, translocations, aneuploidy, high frequency of repetitive sequences, and other anomalies of chromosomes are known to affect recombination levels (24). Meagher et al. (25) pointed out that to assume the average kb/cM ratio for a species applies to all loci would result in tremendous errors in estimating physical distances. However, Shen et al. (16), indicated that the waxy locus of maize maps physically in the same region to which it was genetically mapped.

Because recombinational length in plants and animals is not necessarily closely related to physical distances, the physical mapping will be necessary for the accurate location of cloned loci on chromosomes. Therefore, the present study was undertaken to investigate the problems associated with physically mapping low-copy or unique-sequence genes in rye (Secale cereale) and, where possible, to compare the physical and genetic maps.

MATERIALS AND METHODS

Materials

Plants. S. cereale L. cv. Blanco from the University of Missouri collection was used as the rye in all cases because
the chromosomes involved are easily distinguishable by arm ratios, and it has a uniform standard C-banding karyotype (26).

**DNA Sequences.** The cDNA clone pSc503 was supplied by P. R. Shewry ( Rothamsted Experiment Station, England). pSc503 is a cDNA clone corresponding to a rye γ-selenalin gene (57, 28), and contains a HindIII insert of 900 bp in a pUC8 vector.

**Methods**

**Chromosome Preparation.** The protoplast technique used was that developed by Dille et al. (18) for obtaining protoplasts from fixed root tips of rye. The technique involves digesting fixed root tips with cellulase and pectolyase followed by washing the cells and dropping them onto a slide. The chromosomes of rye were identified after in situ hybridization by their morphology and arm ratios.

**Biotin Labeling of Plasmids.** Nick-translation of the entire plasmid with biotin-11-dUTP was done in an adaptation of the procedure of Rigby et al. (29). One and one-half of plasmid DNA was added to a 50-μl mixture of 50 mM Tris hydrochloride (pH 7.5)/5 mM MgCl2/30 μM each of dATP, dGTP, dCTP, (Pharmacia), and biotin-11-dUTP (Enzo Diagnostics) also containing 20 μg of DNase I (Sigma) and 12 units of DNA polymerase I (BRL). The reaction was incubated at 15°C for 2 hr. Labeling was terminated by adding 5 μl of 0.2 M EDTA (pH 8.0). Labeled probe was purified away from uncleaved nucleotides by passing the reaction through a Sephadex G-50 spin column (30). Incorporation of biotin-11-dUTP was evaluated by means of dot blots developed using a BRL streptavidin–alkaline phosphatase-detection system.

**In Situ Hybridization.** Hybridization of biotinylated probes to protoplast chromosome preparations was performed as suggested in instructions provided by Enzo Diagnostics. Slide preparations were incubated in an RNase A (1 μg/ml)/2× standard saline citrate (SSC) (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) solution at 37°C for 45 min. The slides were then placed in 70% (vol/vol) formamide/2× SSC at 70°C for 3.5 min. Preparations were immediately dehydrated in a series of ethanol washes (70%, 95%, and 100% ethanol at −20°C for 5 min each). Slides were allowed to air dry and then placed in plastic high-humidity chambers. Hybridization mixture was prepared by using a labeling reaction adjusted to 50% (vol/vol) formamide/10% (wt/vol) dextran sulfate/2× SSC and salmon sperm DNA at 0.1 mg/ml. This mixture was denatured in a boiling water bath for 10 min, and then placed on ice until ready for use. A measured amount of hybridization mixture was applied to each chromosome preparation, and coverslips were added. The chambers were placed at 80°C for 10 min to denature the preparations and the probe; they were then transferred to a 37°C incubator for overnight hybridization.

**Detection and Visualization of Hybridized Probes.** After hybridization overnight, the chromosome preparations were subjected to a series of washes. Excess probe was rinsed off with 2× SSC, the slides were placed in 2× SSC at room temperature for 5 min, and then the slides were transferred to 2× SSC at 37°C for 10 min. This procedure was followed by another 5-min room-temperature wash in 2× SSC, then a wash in 0.1% Triton/phosphate-buffered saline (PBS) for 4 min, and a last wash in PBS for 5 min, also at room temperature.

Primary and secondary antibodies were used to amplify the signal from the hybridized biotinylated probes. Goat anti-biotin IgG (Sigma) was applied at a concentration of 30 μg/ml, and slides were incubated at 37°C for 30 min. Unbound antibody was removed by washing in PBS 3 times, 1 min each, at room temperature. Biotin-conjugated rabbit anti-goat IgG (Sigma) was applied in a 1:400 dilution of the supplier’s stock. The slides were then incubated and washed as for the goat anti-biotin treatment.

After application of the secondary antibody, streptavidin–horseradish peroxidase conjugate was used as the reporter molecule. Slides were incubated with 0.7% secondary antibody–horseradish peroxidase (BRL) for 30 min at 37°C. They were then washed in 2× SSC for 5 min at room temperature and then in 0.1% Triton/PBS for 4 min. Visualization of the hybridization complex was accomplished by application of 0.05% dianisidinedibenzene hydrochloride (BRL) and 0.03% H2O2 (Sigma) in PBS. These substances were allowed to react with bound enzyme complex for 5 min at room temperature. Excess substrate was removed from the slides with a PBS rinse, and the chromosome preparations were counterstained in 2% Giemsa (EM Science) for 1 min.

**Physical Mapping.** The arm lengths of each labeled chromosome were measured from a high-resolution television screen with calipers. Each measurement was repeated five times to average out any errors. All measurements were taken from the centromere toward the telomere. The same procedure was used when measuring the physical location of a detection site in that all measurements were made from centromere to the detection site, and five measurements were averaged. Because of slight variations in chromosome condensation from cell to cell and slide to slide, five labeled chromosome spreads from each of three plants were averaged to produce the karyotype and determine the detectionsite locations.

**RESULTS AND DISCUSSION**

**Chromosome Preparations.** In situ hybridization of biotin-labeled pSc503 probe to S. cereale root-tip squash preparations was attempted; however, very little hybridization signal was visible. In contrast, hybridization of pSc503 probe was observed on protoplast preparations of the S. cereale chromosomes (see below). This result is in agreement with other studies (12, 13, 17, 31) and shows that protoplast preparations are superior to root-tip chromosome squashes for use in the detection of single-copy or low-copy number DNA sequences. In addition, the use of phase-contrast microscopy greatly improved visualization of the biotin signal.

**In Situ Hybridization in S. cereale.** Hybridization of pSc503 to the satellite region of 1RS of diploid rye occurred in ~8.5% of the cells analyzed (Fig. 1 and Table 1). The amount of detection is relatively high as compared with the 0.025% labeling of a barley (Hordeum vulgare L.) hordein gene reported by Clark et al. (17). However, Shewry et al. (32) indicated that the Sec-1 locus, located on 1RS and producing 40-kDa γ-selenalins, was multigenic. Generally, only one chromatin was labeled (Fig. 1); however, labeling on only one chromatin rather than on both is uncommon (17, 31). At the present time, the reason for this phenomenon is conjectural; it may be related to different degrees of supercoiling in the two chromatids or to the position of the chromatin in the preparation. In addition, it may be related to the size of the probes; papers showing two hybridization spots have dealt with probes of a much larger size, 7–10 kb and larger (1, 5, 33, 34). When dealing with probes of this size, the chances of visualizing two spots is increased.

pSc503 probe also showed cross-hybridization at a much lower frequency to the rye chromosome arms (1RL and 2RS), where Sec-3, which produces high-molecular-weight selenalin s, and Sec-2, which produces 70-kDa γ-selenalin, respectively, have been genetically located by Shewry et al. (32) (Fig. 1, Fig. 2, and Table 1). In addition, cross-hybridization was observed on rye chromosome arm 6RS, where a selenalin locus has been seen in Secale montanum by Shewry et al. (32) (Fig. 1 and Table 1). These workers regarded this as evidence
of a 2R<sup>mon</sup>:6R<sup>mon</sup> translocation. The cross-hybridization to the three additional sites may have resulted from the relatively low stringency used in the <i>in situ</i> hybridization procedure. Hybridization was detected after incubation in the presence of 50% formamide/2× SSC at 37°C, which was followed by two room-temperature washes and one wash at 37°C in 2× SSC. By contrast, hybridizations of low-copy probes to Southern blots of genomic DNA are often conducted in 50% formamide/6× SSC/0.5% SDS at 42°C, usually followed by a series of reduced ionic-strength washes (from 2× SSC/0.1% SDS to 0.1× SSC/0.5% SDS) at 65–68°C (30). Theoretically, the lower the ionic strength of the solution and the higher the temperatures a hybridized probe is subjected to, the greater the degree of homology required between the probe and its binding site for the probe to remain bound. Thus, under stringency conditions lower than used in Southern analysis, <i>in situ</i> hybridization of pSc503 may have detected rough homology between <i>Sec</i>-1 and the other secalin loci. The cross-hybridization seen indicates that even though the genes involved code for different secalins, which evolutionarily diverged a long time ago, they still retain some degree of sequence identity (27). As can be seen from Table 1 the percentage of hybridization of the pSc503 probe to either <i>Sec</i>-2, <i>Sec</i>-3, or the locus on 6RS was one-half, or less, of that observed for the <i>Sec</i>-1 locus. This may well indicate the degree of homology between the genes.

These results agree completely with those of Shewry et al. (28, 32), who found secalin gene complexes on <i>S. cereale</i> L. arms 1RS, 1RL, 2RS, and 6RS<sup>mon</sup> of <i>S. montanum</i>. The gene found on 6RS of cv. Blanco may be at the same locus as the one on 6RS<sup>mon</sup> (32) because these two chromosomes are largely homologous. The 6RS locus must either be missing in certain cultivars or does not produce a detectable product.
Further screening of *S. cereale* germplasm should answer this question.

The recombinational map of rye chromosome 1R (20) positions both the Sec-3 (35) and the nucleolar organizer region loci very close to the centromere (4.65 cM and 2.65 cM, respectively), whereas the physical map shows them to be some distance from the centromere (Fig. 2). This fact indicates that there is a considerable portion of both arms proximal to the centromere, where very little or no crossing-over takes place (19, 36). It is interesting to note that the discrepancy between the physical and recombinational map location is less for the Sec-3 locus than for the Sec-l locus, suggesting as discussed earlier, that recombinational levels can vary tremendously along and among the chromosome arms. Perhaps these regions of the chromosome contain housekeeping genes or other important gene loci present in combinations vital to the plant, which therefore cannot be permitted to recombine if the plant is to survive.

The Sec-l locus was physically mapped to the satellite region of the short arm of rye chromosome 1R (Fig. 2). Within the limits of detection of the light microscope, the locus was physically mapped to the terminal portion of the satellite near the secondary constriction on 1RS adjacent to the nucleolus organizer region. The recombinational map of Lawrence and Appels (20) showed the *Gpi-R1* locus, previously assigned to that chromosome by Chojecki and Gale (37), to be located on the satellite region of 1RS between the nucleolus organizing region and Sec-l. A more accurate physical picture of the gene location should be obtained by using an electron microscope and pachytene chromosomes.

Both advantages and difficulties are associated with the production of physical maps of chromosomes. The advantages of such studies include the following: (i) unlinked loci can be mapped; (ii) an estimate of the size of an alien insert can be obtained; and (iii) when biotin labeling is used, the *in situ* hybridization technique yields unambiguous results quickly. The principal difficulty is that the physical location of a locus is only as precise as are the techniques for detection and measurement. Physical map accuracy will improve as better chromosome preparations and detection techniques become available.

At present, physical mapping of gene loci to chromosomes by using *in situ* hybridization techniques has several advantages over physical mapping by using chromosome walking techniques. The chromosome walking approach will yield an estimate of the number of base pairs that separate two loci; however, it does not relate to the physical location on the chromosome unless the base-pair link involves a telomere or a centromere. This problem is a major one because most existing plant recombinational maps do not show the location of either centromere or telomere. In addition, the degree to which the linear DNA molecule is condensed along the chromosome length is thought to vary. The molecular approach may also involve a great deal of walking through areas of highly repeated sequences before loci of interest are reached, particularly in plants that have large genomes. As discussed earlier, a chromosome walk using molecular techniques could be very time-consuming because of the existence of areas where little or no recombination (cold spots) occurs. The optimal approach to physically mapping DNA sequences of any kind to plant chromosomes would appear to involve a combination of *in situ* hybridization, chromosome walking, and classical cytotgenetic techniques. Clearly, much work remains to be done before both physical and genetic maps will be routinely used by plant breeders and geneticists involved in gene manipulation.

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