Primer-mediated in situ detection of the B-hordein gene cluster on barley chromosome 1H (physical mapping)

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ABSTRACT  In situ hybridization methods allow the detection of specific DNA sequences on whole chromosomes. The technique has been widely used as a diagnostic and research tool by animal cytogeneticists, for whom detection of unique sequences on mammalian chromosomes is routinely achieved. However, detection of unique sequences on plant chromosomes is less reliable. The recently developed primer-induced in situ hybridization (PRINS) technique allows rapid and reliable in situ detection by the hybridization of primers to denatured target DNA, followed by extension with DNA polymerase in the presence of a labeled nucleotide. The use of short oligonucleotide primers could allow improved penetration of debris and highly condensed chromatin common in preparations of plant chromosomes, thus increasing the sensitivity of in situ detection. The feasibility of this approach is demonstrated by the oligonucleotide primer-mediated detection of the B-hordein gene cluster on a barley chromosome. Applications of the PRINS technique for plant cytogeneticists are discussed.

In situ hybridization is a powerful method for the detection of specific DNA sequences on whole chromosomes. The technique is widely applied in the field of human cytogenetics—e.g., for identification of single chromosomes, chromosomal abnormalities, and localization of repeated and unique DNA sequences to specific chromosome segments (1, 2). In its original form, the technique involves hybridization of a labeled DNA or RNA probe to fixed chromosome spreads. The probe may be labeled radioactively or nonradioactively with nucleotide derivatives detectable directly or indirectly by fluorescence—e.g., rhodamine or biotin/avidin fluorescein conjugates. The chromosome segment where hybridization has occurred can then be identified by the appropriate detection method.

Recently, the development of primer-induced in situ hybridization (PRINS) has been reported (3). The technique involves annealing unlabeled DNA primers (either restriction fragments or oligonucleotides) to complementary sites on denatured chromosome spreads, followed by incubation with DNA polymerase and deoxyribonucleotides. The annealed primers provide initiation sites for synthesis of a new DNA strand by DNA polymerase. Inclusion of a labeled nucleotide in the reaction mixture allows detection of synthesized DNA in situ. The PRINS technique has proved to be a useful tool for human cytogeneticists (4).

Plant cytogeneticists have used conventional in situ hybridization to distinguish between different genomes and for physical mapping of repeated sequences (5). The detection of low-copy sequences, such as the B-hordein gene cluster in barley (6), the secalin gene clusters in rye (7), and a number of unique sequences, also has been reported (8–10). However, efforts to reliably detect unique sequences on plant chromosomes by using labeled DNA probes have a low success rate. The occurrence of cell wall fragments and cellular debris is known to reduce access of DNA probes to target sites on plant chromosomes, thus decreasing the sensitivity of in situ detection (11). Furthermore, detection of certain sequences may be hindered by the presence of highly condensed chromatin, as seen in human and plant chromosome preparations (12, 13). The use of PRINS, with an oligonucleotide as the annealed primer, could allow better penetration of debris and condensed chromatin, thus increasing the efficiency of hybridization and sensitivity of detection.

Previously, PRINS has been used to detect repeated sequences on plant chromosomes (14). This paper demonstrates that PRINS provides a reliable and sensitive method for detection of clustered, low-copy sequences on plant chromosomes using oligonucleotides as the hybridizing reagents. As a test case, the Hor2 locus of barley was selected. Hor2 consists of 10–15 genes encoding the B-hordein seed proteins (15, 16) and has been genetically mapped to the short arm of chromosome 1H (barley chromosome 5), along with a number of loci encoding related, but distinct hordein proteins (17). Previously, Clarke et al. (6) have physically mapped Hor2 using nonradioactive in situ hybridization, thus providing a comparison for these in situ detection experiments. The data show that Hor2 can be physically localized to a specific chromosome arm using PRINS. The potential applications of this technique are discussed.

MATERIALS AND METHODS

Plant Material. Cytological specimens were prepared from barley (Hordeum vulgare L., cv. Golden Promise) and a monotelosomic addition line of hexaploid wheat (Triticum aestivum L., cv. Chinese Spring), containing the short arm of chromosome 1H of Hordeum chilense Roemer and Schultes (18). Seeds were germinated on moist filter paper in Petri dishes. Root tips were removed when 1–2 cm long and treated with an ice-cold saturated aqueous solution of 1-bromo-naphthalene for ~5 hr. Root tips were then fixed in ethanol/acetic acid (3:1).

Probe DNA. Oligonucleotides were synthesized on an Applied Biosystems oligonucleotide synthesizer (model 380A). The oligonucleotide primers Hor2.1 (5'-ATG-TAAAGTGAATAAGGT-3') and Hor2.3 (5'-TACCTGCA-TGGTATTAG-3') were originally designed for PCR amplification of a conserved region of the B-hordein gene family (16). The specificity of the primers was checked by PCR amplification of a single product from barley genomic DNA, and the identity of the PCR product was confirmed by Southern hybridization with a DNA probe derived from a cloned B-hordein gene (data not shown; ref. 16).

Abbreviations: PRINS, primer-induced in situ hybridization; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride.

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Slide Preparation and Pretreatments. Chromosome spreads were prepared on glass slides following Fukui and Jima (19). Chromosome spreads were pretreated with RNase [1 μg/ml in 2× SSC (2× SSC is 0.3 M NaCl/0.03 M sodium citrate)] for 1 hr at 37°C.

It was possible that the procedures used for slide preparation could generate random nicks in chromosomal DNA. Nicks would provide initiation sites for DNA polymerase during subsequent labeling steps, leading to generation of nonspecific background. A nick-filling step was included to avoid this problem (20). Nick-filling was performed by incubating slides for 2 hr in a humid chamber at 37°C with 40 μl of a reaction mixture consisting of 25 μM each dATP, dCTP, dGTP, TTP and 0.1 unit of Klenow DNA polymerase per μl (Northumbria Biotechnology, Cramlington, U.K.) in nick-translation buffer (NT buffer; ref. 21). Lastly, the spreads were treated in 4% paraformaldehyde as described by Heslop-Harrison et al. (22).

Primer-Mediated in Situ Detection. The procedure was modified from the PRINS method of Koch et al. (4). Chromosomes were denatured in 70% formamide in 2× SSC at 70°C for 3.5 min. Slides were then dehydrated in an ethanol series, air-dried for 30 min, and preheated to the annealing temperature (32°C or 42°C) for 20 min in a humid chamber.

When the annealing temperature had been attained, 40 μl of probe mixture was applied to the slide. The probe mixture consisted of between 0 and 500 ng of each B-hordein-specific primer, 10% dextran sulfate, 1% SDS, 0.5 μg of heat denatured salmon sperm DNA per μl, and 2× SSC. Annealing of the primers was carried out for 16 hr. After annealing, slides were washed twice for 10 min in 2× SSC at the annealing temperature. Next, the slides were washed twice for 10 min in NT buffer, then again at the annealing temperature. The labeling mixture was then added, using 40 μl per slide. The labeling mixture consisted of 75 μM dATP, dCTP, dGTP, and 20 μM TTP, 30 μM 12-fluorescein-dUTP (Boehringer Mannheim) or 4-oxadine-dUTP (Amersham) and 0.1 unit of Klenow DNA polymerase per μl prepared in NT buffer. DNA polymerization was carried out for 40 min at the annealing temperature (32°C or 42°C). Slides were then placed in stop buffer (0.5 M NaCl/0.05 M EDTA) and incubated at 65°C for 5 min. Chromosomes were visualized by staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and propidium iodide (when fluorescein was used as the label) or DAPI alone (when rhodamine was used as the label), as described by Heslop-Harrison et al. (21). The spreads were treated with antifade mounting medium (Vector Laboratories) under a glass coverslip and inspected by fluorescence microscopy using an appropriate filter combination.

RESULTS

In an initial experiment, 50 ng of each B-hordein primer was incubated with spreads of barley somatic chromosomes at 32°C. This temperature was chosen on recommendations made by Sambrook et al. (20) for hybridization of oligonucleotides to DNA immobilized on nylon filters. Extension from the annealed primers was carried out with fluorescein-labeled dUTP. Visualization of fluorescein isothiocyanate fluorescence revealed specific labeling of a single pair of chromosome arms (Fig. 1 c and d). Such a pattern obtained using the B-hordein primers suggested that the region labeled was the short arm of chromosome 1H (barley chromosome 5) but that other adjacent loci on the same arm had also been labeled. This is consistent with the presence of other hordein loci adjacent to Hor2 on the same chromosome arm (17).

Previously, the specificity of the primers had been established by PCR analysis with an annealing step of 42°C (see Materials and Methods). It was therefore possible that the lower temperature used during PRINS had allowed annealing of the primers to the other hordein loci adjacent to Hor2.

In subsequent PRINS experiments the B-hordein primers were incubated with barley somatic metaphase spreads at 42°C and extension was carried out with rhodamine-4-dUTP as the labeled nucleotide. Incubation of the chromosome spreads without primers and subsequent extension in the presence of rhodamine-4-dUTP generated little detectable background (Fig. 1 a and b). After incubation and extension with the B-hordein primers, two regions were seen to be labeled, each consisting of a pair of spots (see Fig. 1e). These observations are consistent with specific detection of B-hordein gene sequences on both copies of chromosome 1H and with labeling of sites on both blackrams. The position of the fluorescent signal on the labeled chromosomes was in close accord with the observations of Clarke et al. (6). In a number of experiments, detection of a localized signal as described above was obtained using 5 and 50 ng of each primer per slide. Application of larger amounts of primer (500 ng per slide) resulted in extensive and nonspecific labeling of all 14 chromosomes in almost every cell observed (data not shown).

To verify the identity of the hybridization sites, chromosome spreads were prepared from a monotelosomic addition line of wheat containing the short arm of chromosome 1H, the wild barley H. chilense (18). The short arm of chromosome 1H is homologous to the short arm of chromosome 1H of cultivated barley (18) and carries storage protein genes related to the B-hordein genes in H. chilense. After primer annealing and extension, two distinct sites, each consisting of two spots, were detected in interphase nuclei from the addition line (Fig. 1j). This corresponds to the expected detection of loci homologous to Hor2 on the two chromosomes of the H. chilense telosome. The metaphase cell in Fig. 1 g and h shows a pair of fluorescent sites consistent with the detection of a hordein gene cluster on the two chromosomes of the H. chilense telosome.

DISCUSSION

This paper describes the in situ detection of the B-hordein gene cluster by hybridization with specific oligonucleotide primers using a modification of the PRINS technique of Koch et al. (3). Synthesis of a labeled DNA strand from the sites where primer annealing had taken place allowed the physical location of the gene cluster to be visualized by fluorescence microscopy. The position of the fluorescent signals on barley chromosomes was in accordance with data obtained by conventional nonradioactive in situ hybridization (6).

The PRINS technique has already proved useful for studies of the organization of repeated sequences on human (4) and plant (14) chromosomes. In both cases, the detection was achieved in less than a day. In situ detection of low- and single-copy DNA sequences has proved problematic for plant cytogeneticists. Probes above a certain size may not efficiently penetrate cell wall fragments, cellular debris, and condensed chromatin to reach their target sites. Loss of sensitivity due to inefficient hybridization might be overcome by the use of long tracts of labeled probe DNA. Indeed, single-copy sequences have been detected using labeled DNA fragments of relatively large size—e.g., 13.5 kb (8) and 17 kb (9). Alternatively, the use of short oligonucleotides as the hybridizing reagent could increase sensitivity by improving penetration of the probe DNA to target sequences. We have demonstrated the feasibility of this approach by the in situ detection of the B-hordein gene cluster using PRINS with a pair of specific oligonucleotide primers. This raises the possibility that any DNA region for which oligonucleotide primers are available could be detected by the PRINS technique—e.g., sequenced complementary DNAs, DNA probes for restriction fragment length polymorphisms, etc.
Fig. 1.  (a) Barley somatic prometaphase cell stained and visualized with DAPI. The slide was incubated without primer as described. (b) Cell shown in (a) visualized by fluorescence microscopy to detect rhodamine label. Note absence of detectable fluorescence. (c) DAPI image of barley somatic prometaphase cell from a slide hybridized with the B-hordein primers at 32°C. (d) Cell shown in (c) visualized to detect fluorescein label. (e) An incomplete barley somatic metaphase showing a pair of hybridization sites visualized by rhodamine fluorescence (see arrowheads). (f) Somatic interphase of *H. chilense* 1H<sup>40S</sup> monotelosome addition in wheat. Hybridization site is indicated by an arrowhead. (g) DAPI image of 1H<sup>40S</sup> addition line somatic metaphase showing the telosome off the metaphase plate. (h) Pair of hybridization sites on *H. chilense* telosome visualized by rhodamine fluorescence is indicated by an arrowhead.
Refinement of detection methods for low- and single-copy sequences on plant chromosomes would have wide application. Primers designed using chromosome-specific DNA clones (23) could be combined and used to paint individual chromosomes. Koch et al. (20) have demonstrated that multiple rounds of PRINS can be performed on chromosome spreads, enabling identification of different chromosome pairs with distinct fluorescent dyes. In situ detection of unique or low-copy sequences could be a rapid means of following the fate of specific chromatin segments during the evolution of related species—e.g., wheat and its wild relatives—or during breeding programs. Studies on genome organization would be aided by the ability to determine the relative physical location of sequences along chromosome arms. If the interval between two sequences had been defined genetically (in terms of recombination units) then the relationship between physical and genetic distances along plant chromosomes could be examined. Furthermore, accurate in situ mapping may provide a bridge between long-range maps assembled from pulsed-field electrophoresis data (24) and coarser physical mapping studies using deleted chromosome segments in aneuploid stocks (25). This would have considerable value for the many laboratories interested in map-based isolation of specific functional loci.

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