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Combinatorial labelling of DNA probes enables multicolour fluorescence *in situ* hybridisation in plants

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Abstract: This paper demonstrates a simple but effective use of combinatorial probes to label plant chromosomes by multicolour fluorescence *in situ* hybridisation (FISH). Three different DNA probes were labelled with only two different fluorophores, hybridised to somatic metaphase chromosomes of *Secale cereale* and *Triticum aestivum,* simultaneously visualised, and unequivocally distinguished in a single FISH experiment. Combinatorial labelling can augment karyotypical investigations, physical mapping of chromosomes and other analyses in plants based upon FISH.

Key words: Combinatorial labelling - Multicolour FISH - Plant chromosomes - Plant cytogenetics - Rye - Wheat

Introduction

Fluorescence *in situ* hybridisation (FISH) is one of the most extensively used methods in molecular cytogenetics enabling precise physical mapping of DNA sequences directly along chromosomes. This technique has been applied in plants to determine the chromosomal localisation of both genic and non-genic tandem repeats, like 5S and 25S rDNA, centromeric and telomeric repeats, and a variety of genus- or species-specific DNA sequences [4, 9, 10, 12, 16, 30]. It has significantly facilitated karyotype construction, especially in species with small, morphologically similar chromosomes, and greatly contributed to our understanding of the evolution and organisation of genomes of Eucaryota [11, 34]. *In situ* hybridisation with total genomic DNA as a probe (GISH; genomic *in situ* hybridisation) is also used extensively to distinguish between parental genomes in allopolyploid species [1, 18] and hybrids [3], and to identify intergenomic translocations [19, 31].

The optimisation of hybridisation and detection procedures, the use of prophase chromosomes and extended DNA fibres as FISH substrates, and the use of advanced imaging techniques such as confocal laser scanning microscopy (CLSM) have significantly improved the sensitivity, resolution and specificity of FISH [5, 6, 7, 14, 24, 26]. The commercial availability of a range of different fluorophores has enabled the simultaneous imaging of several target sequences in one experiment. In plants, however, traditionally a maximum of three fluorophores are used simultaneously, which emit either blue (amino-methyl coumarin; AMCA), green (fluorescein and derivatives, *e.g.* FITC) or red light (Texas red, rhodamine, cyanine 3; Cy3). Routine counterstaining of chromosomes with for example DAPI, YOYO or propidium iodide, effectively reduces this number since emission wavelengths of these counterstains interfere with emission wavelengths of the commonly used fluorophores.

Combinatorial labelling, which incorporates more than one fluorophore into a probe, is a mean by which a greater number of chromosome targets may be discriminated. It was recently developed for human cytogenetics and is considered as a breakthrough in clinical diagnostics [22]. Nowadays, combinatorial probes are often resolved by computer-aided separation of spectra (combined binary ratio labelling-fluorescence *in situ* hybridisation; COBRA-FISH, spectral karyotyping; SKY, multi-fluor FISH; M-FISH) which has enabled simultaneous and unambiguous identification by colour of all chromosomes of the human karyotype [25, 32]. Furthermore, it provides a powerful tool for the identification of different chromosomal abnormalities both in clinical specimens and neoplastic cell lines [15, 21, 27, 29], and has facilitated comparative evolutionary studies of mammals by cross-species colour segmenting (Rx-FISH) [20].

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To date, combinatorial labelling of probes has rarely been used for physical mapping of plant chromosomes. Jiang *et al.* [13] successfully mapped three different bacterial artificial chromosomes in rice genome with this approach, and recently Fransz *et al.* [6] used a combinatorially labelled 5S rDNA clone in their study on the organisation of the genome of *Arabidopsis thaliana*. In this paper we describe a simple and efficient technique of multicolour fluorescence *in situ* hybridisation in plants, which allows the use of probes labelled with two standard labels to map simultaneously three different DNA sequences along somatic chromosomes of *Secale cereale* and *Triticum aestivum*.

Materials and methods

Plant material and chromosome preparation. Seeds of Experimental B rye (*Secale cereale*; 2n=2x=14 + Bs) and Lindström wheat, which carries rye B chromosomes as additions (*Triticum aestivum*; 2n=6x=42 + rye Bs), were used in this study. Roots 1.0 to 1.5 cm in length were detached from seedlings and treated with 0.05% aqueous colchicine solution for about 6 h at room temperature in the dark then fixed in 3:1 (v/v) methanol:acetic acid mixture and stored at -20°C. Fixed roots were washed in citric acid-sodium citrate buffer (0.01 M, pH 4.6-4.8) for 15 min and digested in a mixture of 20% (v/v) pectinase (Sigma), 1% (w/v) cellulase (Calbiochem) and 1% (w/v) cellulase "Onozuka R-10" (Serva) for 2 h at 37°C. Root tips were squashed in a drop of 45% acetic acid, coverslips were removed by freezing, the preparations were post-fixed in 3:1 (v/v) ethanol:glacial acetic acid mixture, followed by dehydration in absolute ethanol and air drying.

DNA probes. The following probes were used in this study:

(1) D1100: a 1.1 kb fragment of *Secale cereale* B chromosome-specific subtelomeric repeat sequence [23, 33] was labelled simultaneously and in equal proportions with rhodamine-4-dUTP (Amersham - RNP 2122) and digoxigenin-11-dUTP (Roche - 1093 088). Oligonucleotides and conditions used for PCR labelling were as follows: universal M13 forward (5'-CAG GGT TTT CCC AGT CAC GA-3') and reverse (5'-CGG ATA ACA ATT TCA CAC AGG A-3') sequencing primers, 93°C × 5 min, 35 cycles of 94°C × 30 sec, 55°C × 30 sec, 72°C × 90 sec, 1 cycle of 72°C × 5 min. Composition of labelling mixture is shown in Table 1.

(2) pTa794: 410 bp fragment of 5S rDNA unit isolated from *Triticum aestivum* [8] was labelled with digoxigenin-11-dUTP. Oligonucleotides and conditions used for PCR labelling were the same as for the probe D1100.

(3) 25S rDNA: 2.3 kb *Cla*I fragment of the 25S rDNA ribosomal gene of *A. thaliana* [28] was labelled with rhodamine-4-dUTP using a nick translation kit according to manufacturer's protocol (Roche - 976 776).

(4) Total genomic DNA from *Secale cereale* plants carrying no B-chromosomes sheared by sonication to fragments of an average length of about 3-7 kb. The DNA was labelled the same way as 25S rDNA.

Multicolour fluorescence *in situ* hybridisation. Well-spread chromosome preparations were pretreated with DNase-free RNase A (Sigma - R-5503, 100 µg/ml in 2×SSC; 0.3 M sodium chloride and 0.03 M trisodium citrate dihydrate, pH 7.0) for 60 min at 37°C, then washed three times in 2×SSC for 5 min each and post-fixed in 1% aqueous formaldehyde in 1×PBS buffer (0.01 M sodium phosphate and 0.14 M NaCl, pH 7.0) for 10 min, washed in 2×SSC for 15 min dehydrated in an ethanol series (70, 90 and 100%) and air-dried. All DNA probes were mixed to a concentration of about 100 ng per slide along with 50% deionised formamide, 10% dextran sulphate, 2×SSC and 0.5% SDS. Chromosome preparations and predenatured probe (75°C for 10 min) were denatured together using an *in situ* Thermal Cycler (Hybaid) at 75°C for 5-10 min and then incubated overnight at 37°C. After hybridisation, slides were washed for 10 min in 20% formamide in 0.1×SSC at 42°C followed by several washes in 2×SSC. After blocking in detection buffer (5% non-fat dry milk in 4×SSC, 0.2% Tween 20, Merck - 66368 4B) digoxigenated probes were immunodetected by antidigoxigenin antibodies conjugated with fluorescein isothiocyanate (FITC, Roche - 1 207 741) in the same buffer (antibodies dilution 1:12). After final dehydration preparations were mounted and counterstained in antifade buffer (Vectashield, Vector Laboratories - H-1000) containing 2.5 µg/ml of 4',6-diamidino-2-phenylindole (DAPI; Serva).

Table 1. The composition of the labelling mixture used for combinatorial labelling of the D1100 probe

Reagent	Volume (µl)
SDW	27.50
10× reaction buffer with $MgCl_2$ (Promega)	5.00
dATP (2.5 mM, Promega)	2.00
dCTP (2.5 mM, Promega)	2.00
dGTP (2.5 mM, Promega)	2.00
dTTP (2.5 mM, Promega)	3.25
M13 universal forward sequencing primer (5 pmol/µl)	2.00
M13 universal reverse sequencing primer (5 pmol/µl)	2.00
template DNA (super-coiled or linear)	2.00
digoxigenin-11-dUTP (1 mM, 1/2 of the original volume, Roche)	0.88
rhodamine-4-dUTP (1 mM, 1/2 of the original volume, Amersham)	0.88
Taq polymerase (5 u/µl, in Promega buffer B)	0.50
TOTAL	50.01

Photographic documentation and image analysis. Chromosomes were photographed using Fuji Provia 400 ISO colour reversal film with a camera attached to a Zeiss Axioplan epifluorescence microscope. Images obtained from different colour channels were scanned electronically, processed uniformly and finally superimposed using Micrografx Picture Publisher software.

Results

Figure 1 shows the results of simultaneous triple FISH to the somatic C-metaphase chromosomes of Experimental B rye (A-D) and Lindström wheat (E-H). Figures 1A-C and 1E-G show results from single band-pass filters in blue, red and green channels respectively, whilst D and H present electronically superimposed images combining the three individual channels.

The rye somatic chromosomes shown in Figure 1A bear four distinct red signals (Figure 1B), the two smaller

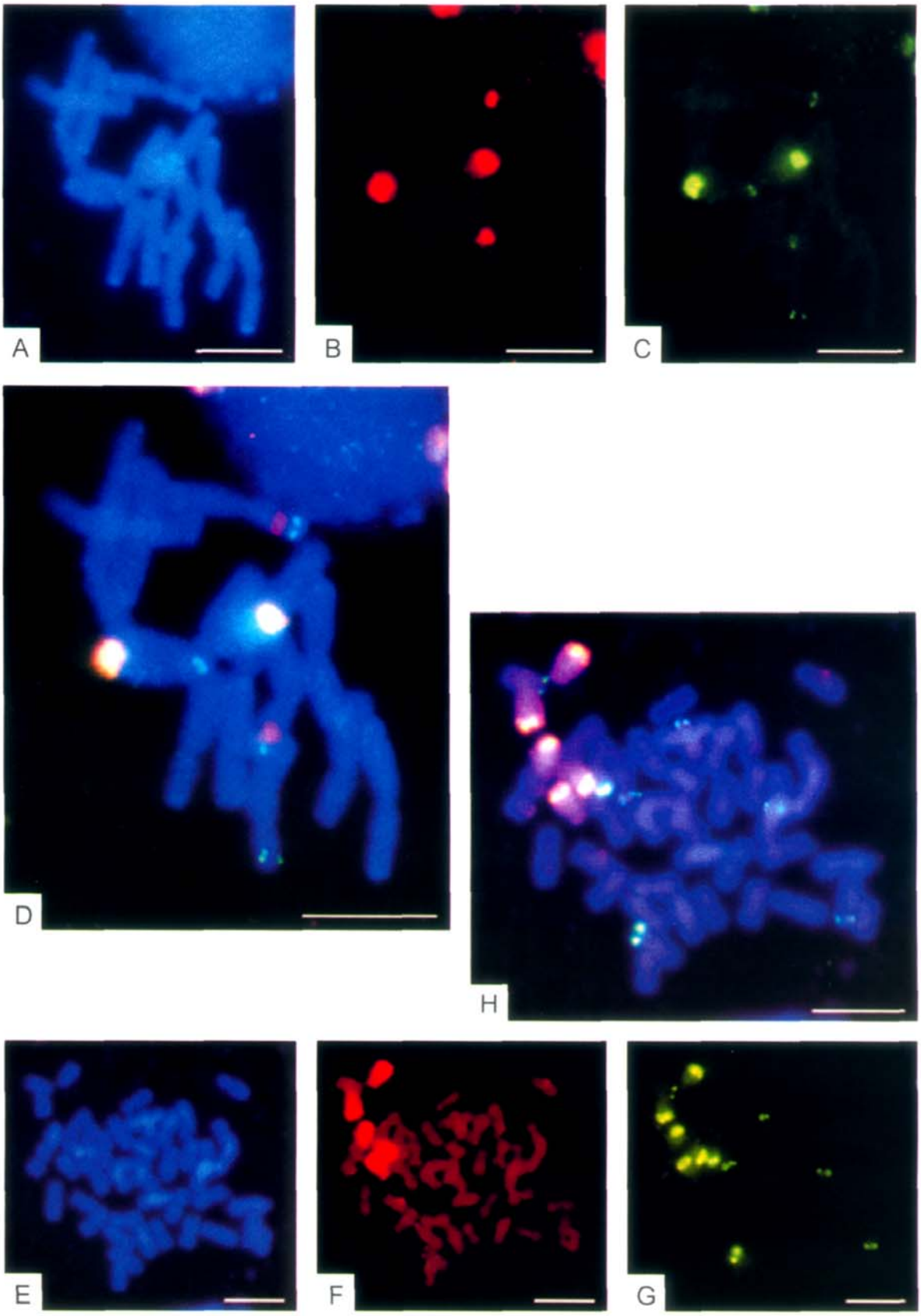

Fig. 1. Simultaneous triple FISH to somatic C-metaphase chromosomes of *Secale cereale* (**A-D**) and *Triticum aestivum* (**E-H**). (**A**) Counterstaining with DAPI (blue). (**B**) D1100 B chromosome-specific subtelomeric sequence (large red signals) and 25S rDNA (small red signals). (**C**) D1100 (large green signals) and 5S rDNA (small green signals). (**D**) Overlay of A, B and C revealing the combinatorially labelled D1100 probe in yellow and clearly distinguishable from the two rDNA sequences in green and red. (**E**) Counterstain with DAPI (blue). (**F**) Rye genomic DNA (dull red) and D1100 (bright red). (**G**) D1100 (large green signals) and 5S rDNA (small green signals). (**H**) Overlay of E, F and G revealing combinatorially labelled D1100 probe in yellow, 5S rDNA in green and rye genomic DNA in pink. Bar = 10 μm.

ones mark the 45S rDNA loci localised at the secondary constriction of chromosome 1R, and the two larger ones highlight the distal heterochromatic segments of the two B chromosomes. Figure 1C shows the same two B chromosome-specific segments fluorescing green, together with four minor signals representing the 5S rDNA loci on chromosomes 1R and 5R. Upon superimposition of the three colour channels, the red and green fluorescence of the B chromosome-specific probe combine to give two bright yellow signals, which are clearly distinguishable from the 45S rRNA and 5S rRNA loci which still appear red and green, respectively. Hence, combinatorial labelling of the B chromosome-specific repeat permits the unequivocal mapping of three sequences with two labels.

Labelled genomic DNA of rye is clearly able to pick out the five B chromosomes in a wheat background (Fig. 1F), which has been used to good effect recently to track B chromatin in interphase nuclei of wheat [17]. Differential labelling within the B chromosomes themselves is also apparent (Fig. 1F), since the red signals of the B chromosome-specific repeat are confined to the distal heterochromatic blocks which fluoresce more brightly. Since the D1100 probe is labelled combinatorially, the heterochromatic segments also fluoresce in the green channel (Fig. 1G), together with seven 5S rRNA loci. The composite image (Fig. 1H) affords better discrimination, since the B chromosome repeat fluoresces yellow, the dull red rye B chromosomes now fluoresce pink against the blue counterstain (Fig. 1E), and the 5S rRNA loci fluoresce green. Thus, the multicolour discrimination of one genomic probe and two cloned DNA sequences is accomplished within one FISH experiment.

Discussion

Simultaneous *in situ* hybridisation of more than two different DNA probes to the same plant chromosome preparation is informative but technically demanding. Hybridisation parameters cannot be optimised for each individual probe, so a compromise of conditions needs to be determined. This problem has been circumvented in the past by sequential *in situ* hybridisation of two or more probes to the same chromosome preparations [2, 3, 26]. Whilst this allows optimisation of conditions for each probe, there is the risk that reprobing of the same preparation may introduce unacceptable background noise, compromise the ultrastructure of the chromosomes or even remove parts of the substrate. More importantly, it is necessary to ensure that the emission spectra of the probes are sufficiently well separated from each other, and from the counterstain of the chromosomes. Interference from the latter can be offset partially by reducing the concentration of the counterstain used. However, optimal concentrations need to be assayed and are often dependent, for example, upon the species under investigation, the preparatory method used for the substrates, and the age of the chromosome preparations. Some newer fluorophores emit in the far-red range of the spectrum, such as cyanine 5 (Cy5) and cyanine 7 (Cy7), potentially increasing the number of useful probes available in a single FISH experiment. However, these fluorophores have technical drawbacks which limit their usefulness, such as their relatively low emission intensity and requirement for non-standard emission filters and digital image capturing devices.

It has been demonstrated above that combinatorial labelling of probes offers a simple, inexpensive and efficient way of discerning more chromosome targets with the same number of fluorophores in one FISH experiment. The method does not require expensive imaging equipment, and is particularly suitable for laboratories equipped with standard fluorescence microscopy only. Combining three different labels in pairwise combination enabled seven probes to be resolved in human metaphase chromosome [22]. There is no reason why this strategy could not be adopted for plant chromosomes too.

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