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Full Length Research Paper

A simple metaphase chromosome preparation from meristematic root tip cells of wheat for karyotyping or *in situ* hybridization

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In this study, a simple and efficient metaphase chromosome preparation method from wheat was described which can also be applied on most other plants. The act of not using hydrolyzing chemicals such as hydrochloric acid in this method made the samples suitable for C-banding and *in situ* hybridization as well. Squeezing out the meristematic cells from the root tip region on to the surface of the slide is the main step of the described method, leading to monolayer and clean preparations. In order to arrest cells in metaphase, colchicine, α-monobromonaphthalene, ice cold water, colchicine-ice cold water and α-monobromonaphthalene-ice cold water were compared to each other. Ice water pretreatment resulted in the highest metaphase index (up to 20% in some cultivars). Some preparations were used for *in situ* hybridization. The described method can be reliably applied in the laboratory with only basic equipments.

Key words: Fluorescence *in situ* hybridization (FISH), karyotyping, squashing method, wheat.

INTRODUCTION

High quality metaphase preparations containing high number of appropriate metaphase spread is a prerequisite for cytogenetic studies such as chromosome banding procedures and *in situ* hybridization. Although the commonly used squash preparation has been argued in different studies (Henegariu et al., 2001; Bertao and Aguiar-Perecin, 2002; Anamthawat-Jonsson, 2003), little has been pointed out about the detailed protocol of making good quality chromosome spreads especially in the field of plant's studies. Among plant tissues containing actively dividing cells, root-tip meristems are the ones most commonly used to make mitotic chromosome preparations. However, other tissues can also be used, such as leaf meristems (Anamthawat-Jonsson, 2003), calli, or protoplasts (Nishibayashi et al., 1989). It is believed that squash techniques require experience and skillful work which yields a relative low fraction good spreads. The difficult preparation of plant metaphases caused by their rigid cell wall and cellular debris usually hampers modern cytogenetic techniques such as genomic and fluorescence *in situ* hybridization. The quality of the preparation is absolutely critical for good hybridization results. The preparation should be well spread, flat and have plenty of chromosomes with good morphology. In addition, the chromosomes should be free from cytoplasmic remains and other cellular material. That is why some researchers have developed expensive protocols based on enzymatic digestion of the cell wall (Shishido et al., 2001; Bowler et al., 2004) or protoplast isolation and drop technique (Busch et al., 1996; Andras et al., 1999). Although there are some publications describing synchronization of plant root tip cells (Pan et al., 1993; Doležel et al., 1999), they are time consuming and expensive for banding and *in situ* hybridization techniques.

In the present paper, the detailed steps of a squash technique have been described that is suitable for karyotyping and *in situ* hybridization. It is a simple technique and can be reliably applied in the laboratories with only basic equipments and little funds. The drawbacks and benefits of some common pretreatments for accumulation of cells in metaphase are also discussed.

MATERIALS AND METHODS

The plant materials used in this study were two synthetic amphiploid lines of Tritipyrum 'Azb' and 'KabCrb' (2n = 6x = 42; AABBE^{b}E^{b})

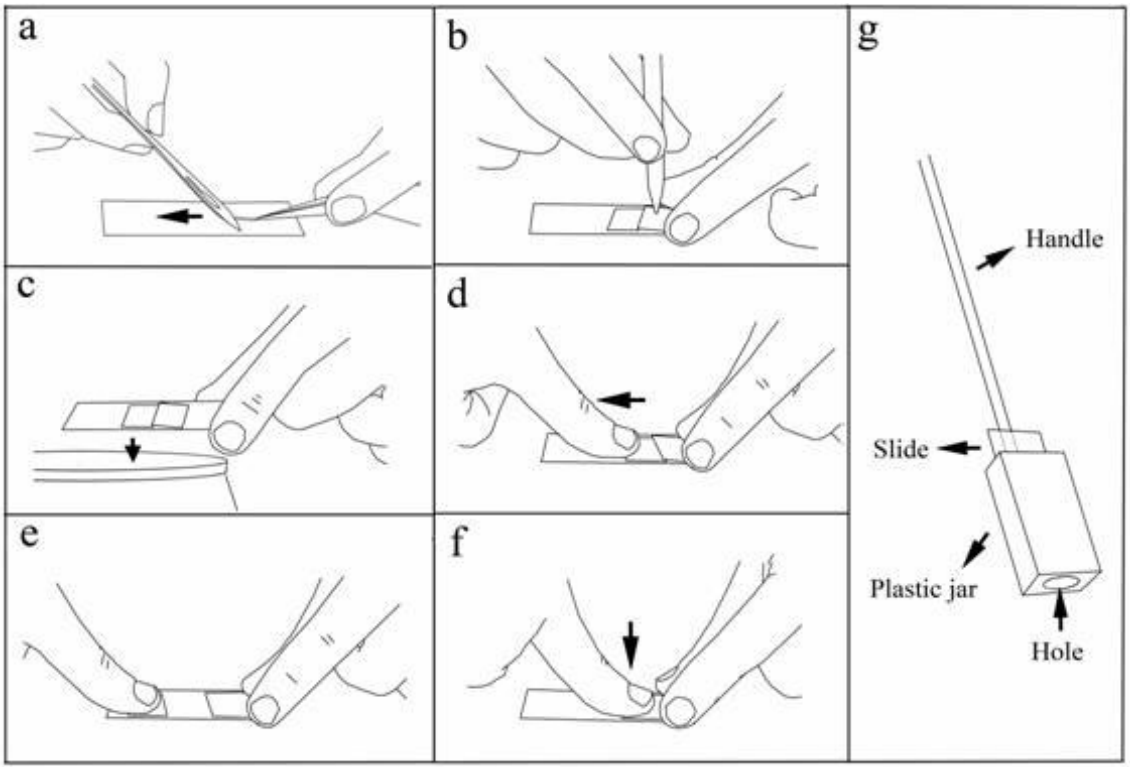

Figure 1. Diagrammatic representation of the method for making mitotic metaphase preparations from root tip cells. For details refer to materials and methods.

and two Iranian bread wheat cultivars 'Roushan' and 'Dez' (2n = 6x = 42; AABBDD). The seeds were put on wet filter paper in Petri dishes and were then placed in a 26°C incubator until emergent roots reached 0.5 to 1 cm long. Roots were cut and divided to 5 ports and each part was then separately pretreated with one of the following pretreatments in order to accumulate mitotic cells in metaphase: (1) 0.05% (w/v) colchicine solution for 3 h at 26°C, (2) saturated aqueous solution of α-monobromonaphthalene for 3 h at 26°C, (3) Ice cold water (0°C) for 24 h, (4) 0.05% (w/v) colchicine solution for 3 h at 26°C followed by ice cold water for 24 h and (5) Saturated aqueous solution of α-bromonaphthalene for 3 h at 26°C followed by ice cold water for 24 h. For ice water treatment the roots were cut and pretreated in tap water in 1.5 ml tubes on ice for 24 h.

Fixation

Pretreated root tips were removed and quickly blot-dried excess water before placing in cold Carnoy's solution I fixative (a mixture of 1 part glacial acetic acid and 3 parts of absolute ethanol which should be prepared fresh daily). For karyotyping or C-banding the materials were fixed for up to two month, for *in situ* hybridization they were fixed for up to 48 h.

Slide preparation

Different steps of slide preparation are diagrammatically presented in Figure 1 which was generated in Adobe Photoshop software according to the actual pictures. At first the fixative were drained and the roots placed in 45% glacial acetic acid for at least 5 min then the following steps were applied: a) the very small root tip cap was cut off with a clean scalpel on a clean slide. The meristematic tissue of root tip squeezed out on to the surface of the slide and 20 to 30 µl of 45% acetic acid was added. In some cases, 45% aceto carmine was added instead of 45% acetic acid. b) A cover slip was placed to one side and another cover slip placed on the squeezed out tissue and the edge of the previous cover slip, then tapped gently with a pencil. c) The slide was heated on a hot heater without boiling the acetic acid. d) and e) The cover slip underneath was removed by pulling toward the far end of the slide while firmly holding the opposite end of the above cover slip. f) The excess liquid was immediately removed with filter paper by gently pressing the cover slip without moving it.

For C-banding or *in situ* hybridization the cover slip was separated from the slide after freezing with liquid nitrogen and the frozen slide immediately immersed in 99% ethanol for the appropriate time. In this study, a special basket for freezing the slides was used (Figure 1g). It has been built up of a handle connected to a plastic jar. The bottom of the jar was punctuated to allow the nitrogen come up in to the jar and freeze the slide when put in the nitrogen tank.

Slides were analyzed with an Olympus BX50 microscope and images of selected spreads were captured using a DP12 digital camera. About 1000 to 2000 cells from each slide were observed and 10 slides were made from each genotype. The ratio of metaphase cells was determined. Metaphase index of the different pretreatments were compared by one-way ANOVA using Minitab software (Ryan and Joiner, 2001) after normality test and Bartlett's test of homogeneity.

Genomic *in situ* hybridization (GISH)

The rye genomic DNA was labeled with biotin-16-dUTP using a nick translation kit (Roche), according to the manufacturer's instructions.

In situ hybridization and detection were carried out as described by Mirzaghaderi et al. (in press) in order to verify the presence of 1RS rye chromosome arm in the 1RS.1BL translocated bread wheat cv. 'Dez'.

RESULTS

Among the pretreatments applied for arresting the cells in metaphase, ice water yielded the highest metaphase index with an average of 19.45% of metaphase cells. This pretreatment always arrest cells in metaphase 3 to 4 times more than colchicine and α-bromonaphthalene (Figure 2, Table 1). There were no significant differences between colchicine and α-bromonaphthalene from the viewpoint of the ratio of metaphase cell accumulation but colchicine pretreatment preserves chromosome morphology better and so is preferred if the chromosomes are to be analyzed by C-banding. Sister chromatids tend to fall apart after this treatment and sometimes are connected only by the centromeres. In the case of long treatments, the centromere was also split. However, no anaphase movement took place and thus the sister chromatids laid close together forming ski pairs (Figure 3). The studied lines and cultivars were also different in response to different pretreatments and cv. Roushan yielded the highest metaphase index in average, that is, 10.72% (Table 1).

DISCUSSION

Chromosome condensation and spreading

It is believed that root chromosomes prepared by conventional squashed technique made poor quality spreads. It was either the chromosomes stick together or some are lost or float away between cells during tapping and squashing (Anamthawat-Jonsson, 2003) while the described protocol here optimized for *Triticum aestivum* allowed the preparation of appropriate metaphase spreads not only for wheat but also for various other cereals including barley and rye without losing the chromosomes. Squeezing out the meristematic tissue on to the surface of the slide presented clean slides. Heating the slide over a heater without boiling the acetic acid helped to break the cell wall and clear appearance of cytoplasm surrounding the chromosomes being almost invisible. The squashing of ice-cold water pretreated materials was easier when compare with colchicine and α-bromonaphthalene giving better metaphase spreads. While colchicine and α-bromonaphthalene was effective in achieving good chromosome morphology, it is advisable to transfer pretreated root tips to ice water for overnight incubation (as in forth and fifth pretreatments of Table 1). This additional pretreatment further enhanced chromosome condensation and more importantly, improved the spreading of chromosomes within a cell. Using colchicine requires determination of optimal concentration for different species. Using lower than optimal concentrations do not arrest cells in metaphase. Optimal concentration (usually 0.05% w/v) acts through depolymerization of the microtubular cytoskeleton in all phases of the cell cycle (Caperta et al., 2006). On the other hand unnecessary high concentration may induce excessive chromosome clumping. These characteristics of colchicine are similar to amiprophose methyl (AMP) which is also a spindle inhibitor (Doležel et al., 1999).

Fixation

The fixation preserves the tissue morphology and minimizes endogenous nuclease activity and other degradation processes. The roots were routinely fixed in ethanol:acetic acid (3:1, v/v). This fixative can also be used for most tissues but is not suitable for microdissection experiments as it cause depurination of DNA and therefore, not suitable for recombinant DNA studies. For recombinant DNA, it is better to use a proper fixative or in the case of using ethanol : acetic acid, the period of the fixation should be shortened to about 30 min (Houben et al., 2001). For fixing oily or waxy tissue, a fresh mixture of Carnoy's Solution II (6 volumes of 100% ethanol : 3 volumes of chloroform : 1 volume of glacial acetic acid) will increase penetration. This fixative is specially preferred for fixing meiotic materials in plants. Both Carnoy's Solution II and Carnoy's Solution I are protein-precipitating fixatives. The other types of fixatives are cross linking fixatives (e.g. glutaraldehyde, formaldehyde) (Leitch et al., 1994), which are usually used for three-dimensional FISH (Bass et al., 1997).

Modifications

Modifications may be applied to some steps of the protocol. For example in plants with tiny roots, it is difficult to squeeze out the meristematic cells from the meristem on to the surface of the slide. Thus, it may be better and easier to cut and use the whole meristem instead of squeezing out the content of the meristem. Even, it is worth trying to squash without using the underneath cover slip in such cases. Another modification is to heat the slide gently after (not before) removing the underneath cover slip and immediately proceed to the next steps. Some laboratories use alcohol burner for heating slides but we found a hot heater safer and easier for this purpose.

FISH applications

To enhance accessibility of the probe and to have a good signal to noise ratio only high quality preparations containing cytoplasm-free and well-spread metaphases

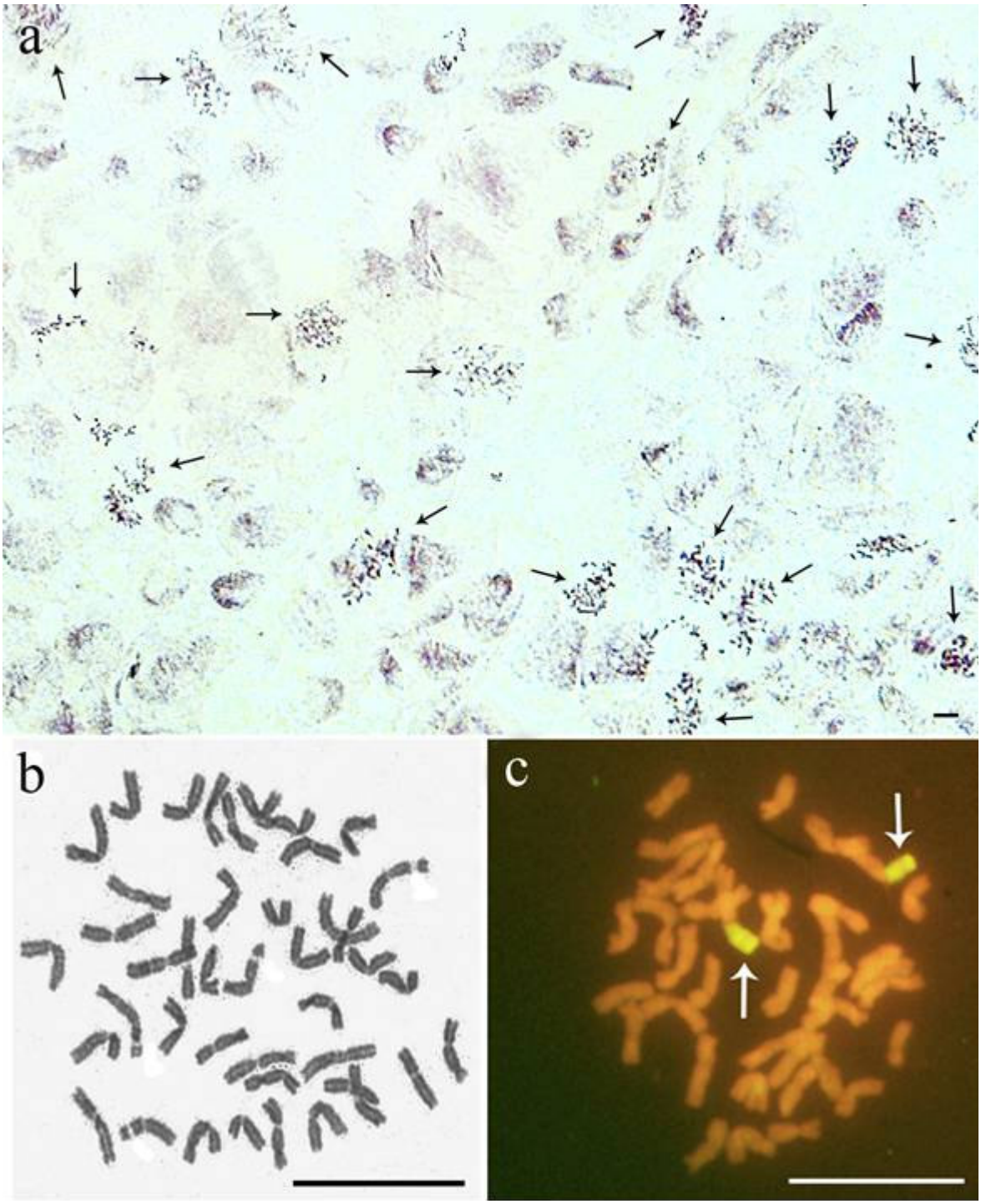

Figure 2. Mitotic metaphase root tip cells of wheat and Tritipyrum; a) Mitotic activity of wheat cv. Roushan root tip cells 24 hours after ice cold water pretreatment, b) Mitotic metaphase spread from a root tip cell of Tritipyrum, c) GISH on metaphase chromosome spreads of 'Dez' using rye genomic DNA probe showing translocated rye chromosome arm. Bar = 20 (µm).

should be used for *in situ* hybridization. The root cap cells and vascular cells have hard cell walls and make squashing difficult. They also prevent the accessibility of a large portion of the probe from the metaphase cells. Squeezing out the meristematic tissue instead of using whole meristem region of the root for squashing excluded most of the undesired differentiated vascular cells and led to the generation of clean slides. Although heating the

Table 1. Mean percent of metaphase index as a result of different pretreatments. Data are based on 10 slides of each cv. or line ±Standard error (SE). The data are based on 10 root tip preparations.

Pretreatment	Genotypes				
	Roushan	Dez	Azb	KabCrb	Mean
Colchicine	5.58 ±0.70	4.45±0.52	6.10±1.60	8.06±1.46	6.048[b]
α-bromonaphthalene	9.67±1.17	5.11±0.98	7.00±1.36	9.83±1.50	7.902[b]
ice cold water	22.08±2.26	22.17±2.94	16.64±3.05	16.91±2.55	19.451[a]
colchicine-ice cold water	6.47±0.97	4.20±0.50	6.25±1.14	5.67±1.41	5.651[b]
α-bromo- ice cold water	9.76±1.26	3.59±0.65	7.07±1.22	8.17±1.27	7.145[b]
Mean	10.712[a]	7.904[b]	8.612[ab]	9.728[ab]	

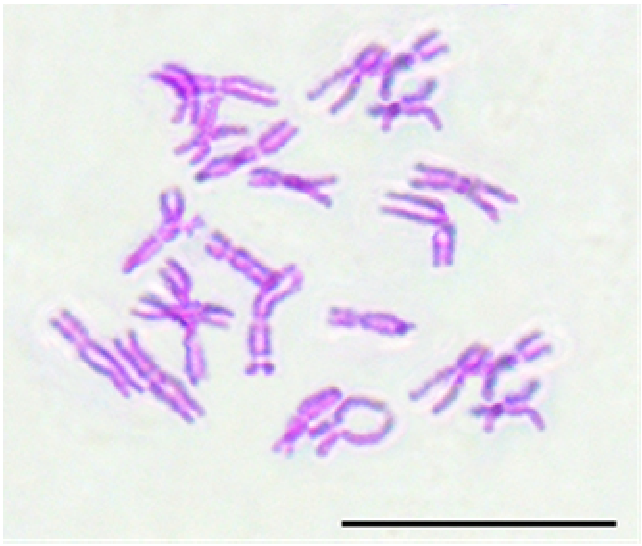

Figure 3. Ski pairs of chromosomes in a partial metaphase spread. This configuration is commonly the result of long pretreatment of the roots with colchicine. Bar = 20 (µm).

slide decrease the cytoplasm segments on metaphase spreads, overheating also reduces the quality of fluorescence signals in FISH.

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