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Differential Screening of Rye-Type cDNAs from a Common Wheat Carrying the Rye Midget Chromosomes

Minoru MURATA

Occurrence of the midget chromosome in a common wheat with rye cytoplasm [(*cereale*)-Chinese Spring (CS)] indicates that the chromosome carries the essential gene(s) for maintaining the function of rye cytoplasm. To elucidate the interaction between the midget chromosome and rye cytoplasm, in this study, an attempt was made to isolate rye-type cDNAs from a cDNA library of (*cereale*)-CS by differential screening. Two replica filters from each plate were hybridized with digoxigenin (DIG)-labeled wheat CS cDNAs and with DIG-labeled rye cDNAs, respectively. Out of ca. 20,000 plaques, 27 were hybridized more strongly with rye cDNAs than with CS cDNAs. These clones were classified into six classes (I-VI) by blot hybridization. The majority of the clones (21 out of 27) was belonged to the same class (1), showing rye-type RFLP (restriction fragment length polymorphism). The DNA sequence of clone CrC1A in class I, was very similar to that of wheat ribulose 1,5-bisphosphate carboxylase, large subunit gene, *rbcL* (95.4% homology). However, the 3' end of the CrC1A was shorter than that of wheat *rbcL*, and terminated at TAA instead of TAG, like the *rbcL* of *Aegilops crassa*. In the clone CrC5.4, the first half of the sequence was similar to that of one rice EST clone, the functions of which are not known, and the latter was similar to the reverse sequence of maize 4.5S-23S ribosomal RNA. This suggests that CrC5.4 had been derived from two different cDNAs of (*cereale*)-CS. Three other clones had homology to the chlorophyll a/b binding protein genes (*cab*) of wheat, maize and tomato, and one to wheat *rbcS* (ribulose 1,5-bisphosphate carboxylase small subunit gene). However, no clear polymorphisms were detected between wheat and rye by using those clones as probes.

Key words : Cytoplasm substitution line, Differential screening, Midget chromosome, Rye, Wheat

Research Institute for Bioresources, Okayama University, Kurashiki 710-0046, JAPAN
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INTRODUCTION

Common wheat with a rye cytoplasm is relatively vigorous compared with other cytoplasm substitution lines of common wheat (Mann and Lucken 1971, Tsunewaki 1980). However, this does not necessarily mean that rye cytoplasm is similar to that of common wheat and there is no incompatibility between wheat nucleus and rye cytoplasm. In fact, Murai et al. (1989) showed that there are differences in chloroplast DNA structures between common wheat and rye. Nakata et al. (1986) found an extra telocentric chromosomes in the rye cytoplasm substitution line, (*cereale*)-Chinese Spring (CS), and named it "midget" owing to its small size. Transmission of this midget chromosome is limited to some extent from the male side. Seeds lacking the midget chromosome are not germinable because of endosperm abortion. Even if they germinate, the plants are very weak and highly male-sterile (Murata, unpublished data). This indicates that the midget chromosome originated from a certain part(s) of rye chromosome and carries an essential gene(s) for rye cytoplasm.

The rye-origin of the midget chromosome was clearly shown by fluorescence *in situ* hybridization (FISH) with rye genomic DNA as a probe (Murata et al. 1992). Furthermore, it was also shown that the chromosome carries 120-bp repetitive DNA sequences of rye (Murata et al. 1992, Murata 1995). Recently, other repetitive DNA sequences of rye were identified to exist in the midget chromosome (Kota et al. 1994, Francki and Langridge 1994).

Although information on the molecular structure of the midget chromosome has accumulated, no clues have been obtained yet to elucidate the mechanism for the close interaction between the midget chromosome and rye cytoplasm. In this study, therefore, I attempted to isolate specific genes expressed preferentially in (*cereale*)-CS, whose DNA sequences are more similar to those of rye than those of common wheat.

MATERIALS AND METHODS

1. *Plant Materials*

A common wheat (*Triticum aestivum*) cv. Chinese Spring (CS), and its rye cytoplasm substitution line (*cereale*)-CS, and rye (*Secale cereale*) cv. Petkus were used in this study.

2. *RNA isolation*

Total RNAs were isolated from 2 g of 2-week-old seedlings of (*cerelae*)-

CS, normal CS and rye according to the method by Verwoerd et al. (1989). From the total RNA, mRNAs were purified through oligo(dT)-cellulose spun columns (Pharmacia).

3. *cDNA probes*

cDNAs from poly A^+ mRNAs of normal CS and of rye (Petkus) were synthesized by the Uni-Amp Plus Kit (Clonetech). After ligation of the Uni-Amp adaptors to blunted cDNAs, 0.5 ng of the cDNAs were used directly for PCR amplification in a 50 μl reaction mix, consisting of 10 mM TrisHCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, and dTTP, 500 nM Uni-Amp primer (Clonetech), and 1 unit AmpliTaq DNA polymerase (Perkin-Elmer). For digoxigenin (DIG) labeling, the PCR DIG labeling mix containing DIG-11-dUTP (Boehringer Mannheim) was used instead of the dNTP mixture described above. DNA amplification was carried out with the Perkin-Elmer DNA Thermal Cycler (Type 480) by 35 cycles of 1 min at 94°C for denaturing, 1 min at 60°C for annealing, and 2 min at 72°C for extension.

4. *Construction of cDNA library from (cereale)-CS*

Construction of a cDNA library and the following differential screening were schematically presented in Fig. 1. A cDNA library was constructed from mRNAs of (*cereale*)-CS by using the ZAP-cDNA Synthesis Kit (Stratagene) and the Uni-ZAP XR vector (Stratagene) according to the supplier's protocol. The titer of this library was approximately 50,000 pfu/μg of vector arms before amplification. About 20,000 plaques were plated, and transferred to nylon membranes (Hybond N^+, Amersham), two from each plate. One of the membranes was hybridized with DIG-labeled cDNAs of CS, and the other with DIG-labeled cDNAs of rye. The concentration of the DIG-labeled probes was ca. 200 ng/ml, which was estimated from the DIG-labeled control (Boehringer Mannheim). To enhance the hybridization specificity, non-labeled amplified rye cDNAs and CS cDNAs were added to the DIG-labeled CS cDNA and to DIG-labeled rye cDNAs, respectively, at the concentration of 4 μg/ml.

5. *Genomic Southern blot hybridization*

Genomic DNAs were isolated from 2-week-old seedlings of normal wheat CS, (*cereale*)-CS and rye (Petkus) according to the method of Murry and Thompson (1980) with modifications. About 2 g leaves of each cultivar were frozen in liquid nitrogen and pulverized to a fine powder with a mortar and pestle. The frozen powder (1.5 g) was transferred to a 50 ml conical

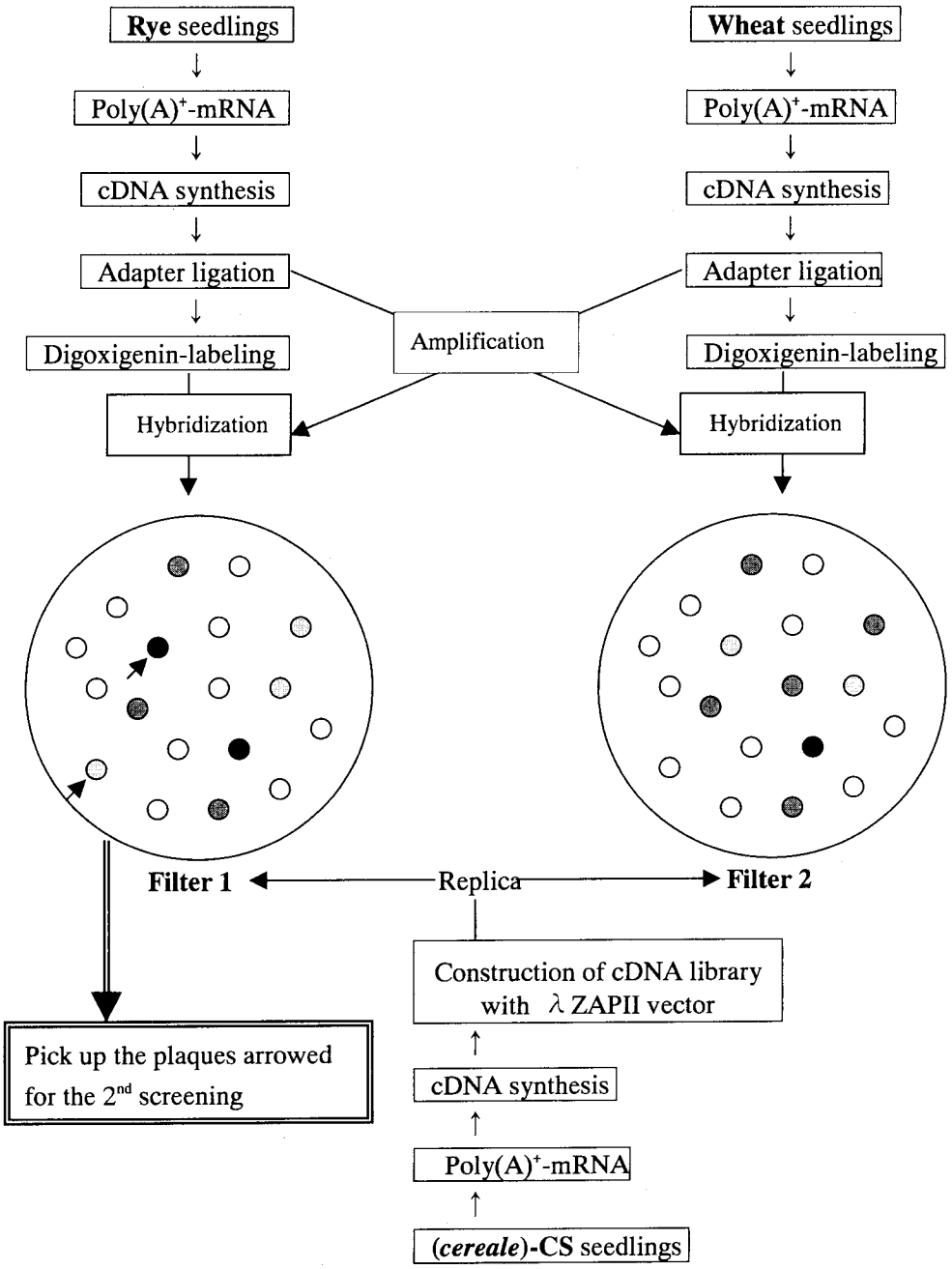

Fig. 1. A schematic representation showing a competitive differential screening of rye-type cDNAs from a cDNA library of (*cereale*)-Chinese Spring (CS).

screw-cap tube, containing 12 ml extraction buffer [50 mM Tris-HCl, pH 8.0, 0.7 M NaCl, 10 mM EDTA (ethlenediaminetetraacetic acid), 1% (w/v) CTAB (hexadecyltrimethyl-ammonium bromide)]. After mixed well with a sterilized spatula, lauryl sarkosyl and Proteinase K (Merck) were added to the solution at the final concentrations of 1% (w/v) and 1 mg/ml, respectively. The mixture was incubated at 50°C for 2 hrs with gentle shaking. After phenol/chloroform extraction and ethanol precipitation, DNA was spooled out by a sterilized glass rod, and transferred to a 15 ml conical tube with 4 ml TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNA was precipitated again with ethanol, and dissolved in 2.3 ml TE, and purified by CsCl gradient centrifugation (Sambrook et al. 1989).

Ten micrograms each of the isolated DNAs was digested with five different restriction enzymes (*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII), and run in 0.7% (w/v) agarose gel overnight. After electrophoresis, DNAs in the gel were stained with 1 μg/ml ethidium bromide, and transferred to nylon membranes (Hybond N^+, Amersham) according to the method by Kreike et al. (1990). The nylon membranes were baked at 120°C for 30 min. As described by Engler-Blum et al. (1993), hybridization was performed at 68°C in 20% SDS (sodium dodecyl sulfate) hybridization buffer with DIG-labeled DNA probes, and chemiluminescence signals were detected on X-ray film (Fuji RX). DIG-labeled probes were prepared from the *Eco*RI-*Xho*I fragments of the selected cDNA clones according to the manufacturer's instruction.

6. *DNA sequencing*

cDNA clones in the ZAPII vector were subcloned into the pBluescript SK(-) by in vivo excision (Stratagene). Using the Dye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer), nucleotide sequences of the inserts in the clones were determined by an automated DNA sequencer (373A, Perkin-Elmer). Determined sequences were analyzed by the GENETYX software (Software Develop.) and BLAST (Altschul et al. 1990).

RESULTS AND DISCUSSION

1. *Differential screening of rye-type cDNAs*

Out of ca. 20,000 plaques plated, 27 clones were preferentially hybridized with labeled rye cDNAs (Fig. 2). Those clones were also hybridized with CS cDNAs, but their signals were much weaker than when hybridized with rye cDNA. To enhance the hybridization specificity, about twenty times of competitive non-labeled CS cDNAs were added to the labeled rye cDNAs

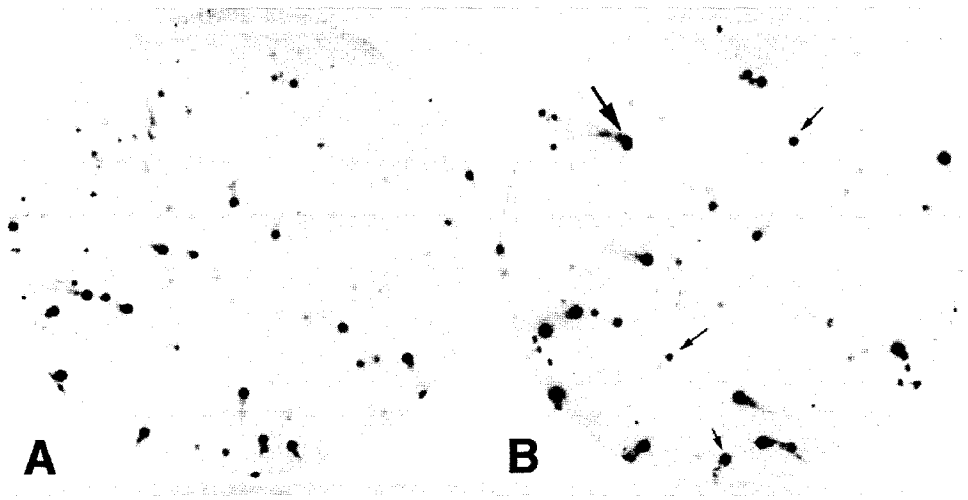

Fig. 2. Detection of chemiluminescent signals on nylon membranes hybridized with DIG-labeled cDNAs of CS (A), and with DIG-labeled cDNAs of rye (B). Arrows indicate plaques showing stronger signals when hybridized with rye cDNAs than with CS cDNAs.

Table 1. Number, size and similar genes of clones screened from a (*cereale*)-CS cDNA library, which hybridized preferentially to rye cDNAs

Class of clones	No. of clones obtained	Clone	Cloned Size (kb)	Similar gene (% homology)
I	21	CrC1A, etc.	0.4−1.5	Wheat *rbcL*[a] (95.4%)
II	1	CrC5.4	0.6	Chloroplast ribosomal 4.5S-23S RNA (96.7%)
III	1	CrC5.2	0.9	Tomato *cab-8*[b] (68.7%)
IV	1	CrC5.3	0.9	Maize *cab-1*[b] (88.9%)
V	1	CrC5A	1.1	Wheat major *cab*[b] (85.5%)
VI	1	CrC5B	0.7	Wheat *rbcS*[c] (93.5%)

a) ribulose 1,5-bisphosphate carboxylase large subunit gene, b) chlorophyll a/b binding protein gene, c) ribulose 1,5-bisphosphate carboxylase small subunit gene.

and vice versa. This addition of non-labeled competitive cDNAs reduced the number of the positive signals to about one twentieth.

The 27 clones screened were classified into six classes (I-VI) on the basis of their homology shown by Southern blot hybridization (Table 1). Twenty-one of them were hybridized to each other and therefore were classified into class I, although the size and the restriction enzyme sites were different among the clones. Eight clones of class I were 1.5 kb in length, and had an *Eco*RI site within the sequences, whereas other clones were 0.4-1.1 kb, and had no *Eco*RI site. One clone (CrC1A) of this class was hybridized to the genomic DNA from CS, (*cereale*)-CS and rye, which were digested with *Bam* HI, *Eco*RI and *Hin*dIII. In all digests particularly with *Eco*RI, there were

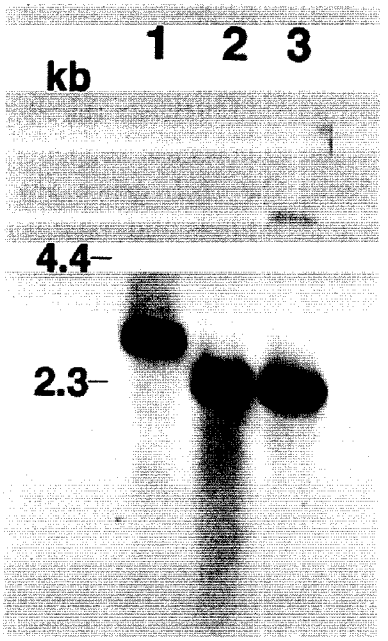

Fig. 3. Southern hybridization patterns of normal CS (1), (*cereale*)-CS (2) and rye (3) DNAs digested with *Eco*RI. The probe used was an *Eco*RI-*Xho*I fragment of clone CrC1A.

restriction fragment length polymorphisms (RFLPs) (Fig. 3). The band patterns in (*cereale*)-CS and rye were identical, but different from that of normal CS. This indicates that the clones of this type had originated from a cytoplasmic parent, rye. However, there was only one band in each lane. Since CS is a hexaploid wheat (2n=6x=42), if it had derived from a specific chromosome, more bands would be expected. Northern blot hybridization with this clone showed no difference among those three lines (no data shown).

The remaining six clones showed no distinct polymorphisms among CS, (*cereale*)-CS and rye, when they were used as probes in Southern blot hybridization.

2. *Sequence analysis*

Partial DNA sequences of six clones, one from each class, were determined. The clones of class I and II were shown to be derived from chloroplast DNA. Homology search by the BLASTN (Altschul et al. 1990) showed that the sequence of CrC1A in the major class (I) was very similar to that of common wheat gene *rbcL* (ribulose 1,5-bisphosphate carboxylase, large subunit) (Terachi et al. 1987). However, the 3' end of this clone terminated as TAA instead of TAG of common wheat, as descibed in *Aegilops crassa rbcL* (Ogihara et al. 1991). This suggests that rye has L-type *rbcL* rather than

the H-type in common wheat, and that the downstream sequences are variable as shown in *Ae. crassa* (Ogihara et al. 1991). Murai et al (1989) indicated that there is an insertion between the *rbcL* and *psa* genes. Murata et al. (1991) also isolated the rye-specific sequences from chloroplast DNA between those genes. Since the *rbcL* genes are known to be expressed abundantly in cells of green seedlings and have AT-rich sequences at the 3' (Terachi et al. 1987), it is reasonable that the majority of the clones screened was from the *rbcL*. Between the sequences of wheat and rye *rbcL* genes, however, there was no large difference (95.4% homology in 854 nt) (Table 1). The sequence of CrC1A was determined only in part. To show the effectiveness of the differential screening method employed here, it is necessary to reveal the difference between the *rbcL* genes of wheat and rye.

The clone of class II (CrC5.4) contains 1.1 kb insert, the sequence from 87 to 344 of which had 85.5% homology to that of an EST clone of rice (Sasaki et al 1994), and that of minus strand from 873 to 1078 had 96.7% homology to that of maize chloroplast 4.5S-23S ribosomal RNA genes. In this clone, no poly-A tail was found, and the last half sequence at the 3' end seems reverse. This suggests that this cDNA resulted from a tail-to-tail fusion between two different cDNAs. So far, no other reason was found why the chloroplast ribosomal RNA without poly-A tail was converted to cDNA and cloned.

Other clones were shown to be derived from the nuclear genome not from the cytoplasmic genome. The clone CrC5.2 (class III) had a relatively high homology (84.8%) to the rice EST clone (S107594A) (Sasaki et al. unpublished), but the function was not clear. Since 68.6% homology was also found to the tomato chlorophyll a/b binding protein gene, *cab-8* (Pichersky et al. 1989), the CrC5.2 possibly belongs to one of the *cab* gene family. Other clones which have more homology to the *cab* genes, were also isolated. The clone CrC5.3 had 88.9% homology to the maize *cab-1* gene (Sullivan eta l. 1989), and the CrC5A showed a high homology (85.5%) to the wheat major *cab* gene (Lamppa et al. 1985). The sequence of clone CrC5B (class VI) was very similar (93.5%) to that of wheat ribulose 1,5-bisphosphate carboxylase small subunit gene, *rbcS*. Since the *cab* and *rbcS* genes form multigene families, and are expressed abundantly in green tissues, they have been easily cloned. The midget chromosome has been shown to be derived from chromosome 1 of rye, which is equivalent to the homoeologous group 1 in other Triticinae species such as wheat and barley. The *rbcS* gene has been mapped on the chromosomes of homoelogous group 2 (Nelson et al. 1995). The *cab-2* genes have been mapped on the homoeologous group 1 of wheat

and barley (Van Deynze et al. 1995), but the position is far from the centromere.

In the differential screening employed here, the clones homologous to the *rbcL*, *cab* and *rbcS* genes, hybridized preferentially to rye cDNAs. This might be due only to abundance of those mRNAs expressed in the young green seedlings, although a rye-type polymorphism was found in the clones of class I (CrC1A and others). To find rare mRNAs expressed from the midget chromosome, therefore, a normalized cDNA library and probes should be constructed (cf. Soares et al. 1994).

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ライムギ小型染色体を保持する普通系コムギからのライムギ型cDNAのディファレンシャルスクリーニング

村田　稔

ライムギの細胞質を有する普通系コムギ(*cereale*)-Chinese Spring (CS)には，「ミジェット」と呼ばれる非常に小型の染色体が存在する。この染色体が失われると，その種子は胚乳が退化し発芽できない。かりに発芽しても，その植物は虚弱で不稔となる。このことは，この染色体にライムギ細胞質の機能維持に不可欠な遺伝子が座乗していること示唆している。本研究では，ディファレンシャルスクリーニング法により，このミジェット染色体に座乗している遺伝子の同定を試みた。(*cereale*)-CSの若苗からcDNAライブラリーを作成し，約20,000のプラークをスクリーニングした。その結果，ライムギのcDNAには強くハイブリダイズするが，CSのcDNAには弱くハイブリダイズするクローンが27得られた。これらについてその特異性をサザンハイブリダイゼイション等で解析したところ，19個のクローン（CrC1Aなど）はサイズ，制限酵素部位に違いは認められるもののお互いにハイブリダイズすることから，同じクラスIに分類された。これらは，正常CSと(*cereale*)-CSとの間でRFLP(制限酵素断片長多型)を示し，その型はライムギと同様であった。このcDNAの塩基配列は，コムギ葉緑体DNA遺伝子*rbcL*に類似しており，ライムギの葉緑体DNAに由来していると考えられた。他のクローンは，コムギの核遺伝子*cab*と*rbcS*にその塩基配列が類似していたが，はっきりとした多型は検出されなかった。ここで用いた方法では，光合成に関連した発現量の多いcDNAがスクリーニングされやすい傾向があり，効率的なスクリーニングには均一化したcDNAを用いる必要があると思われる。

キーワード：細胞質置換系統，ディファレンシャルスクリーニング，ミジェット染色体，ライムギ，コムギ