

## Direct Amplification of an $\alpha$ -Amylase Gene from Barley Chromosome 6 by a Microdissection Method

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### ABSTRACT

Laser microdissection and subsequent direct DNA amplification methods are both effective for plant genome research. By using these techniques, we isolated barley chromosome 6 and amplified the target gene,  *$\alpha$ -amy1*. Barley  *$\alpha$ -amy1* gene, encoding the high-pI  $\alpha$ -amylase, has been mapped onto the long arm of chromosome 6 by Southern hybridization and isozyme analysis using lines with specific chromosome constitution. The primers based on the  *$\alpha$ -amy1* sequence were used for PCR with the isolated chromosomes. When the five microisolated chromosome 6 were used as the template, expected fragments with 142 bp were amplified. By contrast, no PCR product was detected when five chromosome 7 were used as a negative control. As a result, we directly confirmed that the  *$\alpha$ -amy1* gene locates on barley chromosome 6, and concluded that five microisolated chromosomes are sufficient for direct amplification of the target gene.

### Key words

Direct amplification,  $\alpha$ -Amylase gene, Barley, Microdissection method

### Introduction

The microscope-oriented isolation techniques for chromosome (or chromosome fragment) are useful in genome research. Isolated chromosomes can be used as a template for the polymerase chain reaction (PCR) in order to clone the gene in question using sequence specific or random primers. By using such a technique, we could confirm the physical localization of a target gene on the defined chromosome. The construction of chromosome specific library from isolated chromosomes has also been reported (Albani *et al.* 1993, Chen and Armstrong 1995). The chromosome specific library enables the screening of genes

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whose chromosomes are known much easier than that from total genomic library and facilitates the isolation of molecular markers suitable for physical mapping. Furthermore, the above-mentioned direct-cloned DNA and the chromosome specific library would be used as the probe for chromosome specific sequences in fluorescence *in situ* hybridization (FISH) which is an effective approach for gene mapping (Kamisugi and Fukui 1996, Ohmido *et al.* 1998).

For isolation of plant chromosome (or chromosome fragment), following three types of techniques are available at present. First, a flow-sorting method has an advantage for easy separation of large quantities of chromosomes. It has, however, a disadvantage for separation of plant chromosomes with similar size or DNA content (Wang *et al.* 1992, Macas *et al.* 1993a). Second, a micromanipulation method using a glass needle is effective for collection of the small chromosomes, but it needs a skill for handling of the glass needle and is time-consuming (Macas *et al.* 1993b, Sorokin *et al.* 1994). When compared with these two techniques, the third laser dissection method is more effective for chromosome isolation, because we need no special skill (Fukui *et al.* 1992, Kamisugi *et al.* 1993, Sawahel and Fukui 1995).

In this report, we established the direct amplification method for the target gene of relatively small copy number from isolated plant chromosomes by using the laser dissection technique. We selected a barley  $\alpha$ -amylase gene for the model of direct amplification (Khursheed and Rogers 1988).  $\alpha$ -Amylases (EC 3.2.1.1.) are well-characterized endoglycolytic enzymes that play a crucial role for cereal seed germination (Mitsunaga and Yamaguchi 1993, Mitsunaga *et al.* 1994). Barley  $\alpha$ -*amy1* gene, encoding the high-pI  $\alpha$ -amylase, has been mapped onto the long arm of chromosome 6 by Southern hybridization, isozyme analysis and PCR polymorphism using lines with specific chromosome constitutions (Brown and Jacobsen 1982, Muthukrishnan *et al.* 1984, Weining *et al.* 1994). It was also reported that chromosome 6 carries six or more genes closely related to the high-pI cDNA clone (Muthukrishnan *et al.* 1983, Muthukrishnan *et al.* 1984).

## Materials and methods

### *Plant material and cytological technique*

Seeds of barley (*Hordeum vulgare* L.,  $2n = 14$ ) cv. Minorimugi were sown in Petri dishes and were germinated at 25°C in the dark. Root tips 1-2 cm long were excised and pretreated with distilled water at 0°C for 12-24 h to accumulate the metaphase chromosomes. Then they were fixed in a fluid (ethanol and acetic acid, 3:1) for 30 min at room temperature.

After washing in distilled water, the meristematic tips were cut off and macerated using an enzyme mixture of 2% Cellulase Onozuka RS, 1.5% Macerozyme R200, 0.3% Pectolyase Y-23 and 1 mM EDTA (pH 4.2) at 37°C (Fukui *et al.* 1992). After 50 min maceration, the root tips were briefly washed in distilled water and spread over a 35 mm

polyester film-lined culture dish (Meridian Instruments). Three or four root tips were spread on single dish. Air-dried preparations were stained with 1 ml of a 2% Giemsa solution for 30 min, washed with distilled water quickly, and air-dried. Then the dish was subjected to laser dissection immediately or stored in a freezer at  $-20^{\circ}\text{C}$  until use.

#### *Procedure for microdissection and PCR*

A well-spread set of metaphase chromosomes was irradiated with a computer-controlled microbeam of argon-ion laser (488 nm) using the Cell Workstation (ACAS 570, Meridian Instruments, Fukui *et al.* 1992). For the isolation of target chromosome 6, all the chromosomes except the chromosome 6, nuclei and the cell debris were completely ablated by laser irradiation. Barley chromosome 6 is easy to distinguish from the other chromosomes because of the large satellite (Fukui and Kakeda 1990). Then an octagonal polyester disk (about 700-1000  $\mu\text{m}$  in diameter) on which the microisolated chromosomes 6 located, was cut out from the film and the disks dissected were collected in 0.5-ml microtube by the method described previously (Fukui *et al.* 1992, Kamisugi *et al.* 1993, Ohmido and Fukui 1995).

Collected chromosomal samples were incubated with a 20  $\mu\text{l}$  of proteinase solution (1 mg/ml proteinase K, 0.45% Tween 20, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X100) through 6 thermal cycles consisting of  $55^{\circ}\text{C}$  for 30 min and  $37^{\circ}\text{C}$  for 30 min. After heat-inactivation of proteinase ( $95^{\circ}\text{C}$  for 10 min), the 80  $\mu\text{l}$  of PCR reaction mixture was added. Then the final concentration of compounds in the 100  $\mu\text{l}$  solution was 0.2 mM dNTPs, 1  $\mu\text{M}$  AMY1 forward and reverse primers, 1.5 mM  $\text{MgCl}_2$ , 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.09% Tween 20, 0.02% Triton X100 and 2.5 units of Taq DNA polymerase (Promega). Both AMY1 forward and reverse primers were  $\alpha$ -amyl sequence specific primers which were designed according to published genomic sequences of high-pI  $\alpha$ -amylase gene, Amy6-4 (Khursheed and Rogers 1988).

The first PCR reaction commenced with 3 min at  $94^{\circ}\text{C}$ , followed by 30 cycles of 1 min at  $94^{\circ}\text{C}$ , 2 min at  $37^{\circ}\text{C}$  and 2 min at  $72^{\circ}\text{C}$ , terminated with 10 min at  $72^{\circ}\text{C}$ . Then a 5  $\mu\text{l}$  aliquot of the solution was added to the 40  $\mu\text{l}$  of the second reaction mixture. The constituents were the same as the first compounds. The thermal cycle profile was identical with the first reaction except the annealing temperature of  $55^{\circ}\text{C}$ . The PCR products were separated on 2.0% agarose gel in TAE buffer.

## Results and discussion

#### *Direct amplification of $\alpha$ -amy1 gene from isolated nuclei*

Barley  $\alpha$ -amy1 gene, encoding the high-pI  $\alpha$ -amylase isozyme, was selected as a target gene for direct amplification. The predicted length of the PCR product is 142 bp.

We first established the conditions, which allowed specific amplification of the  $\alpha$ -amy1 gene. As the target, we used either the total genomic DNA or microisolated nuclei.

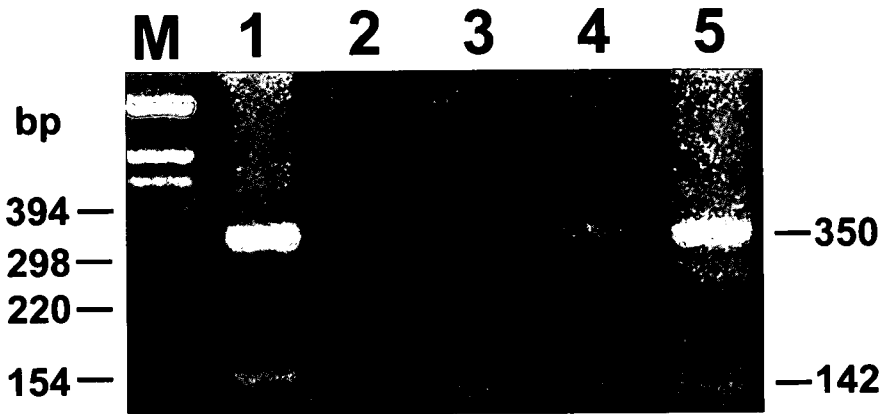


Fig. 1. Agarose gel electrophoresis of PCR products obtained with barley  $\alpha$ -amylase sequence specific primers and template DNAs. Lane M is DNA molecular marker VI (Boehringer Mannheim). Lane 1 is amplified product from total genomic DNA. Lane 2 is the result product without DNA amplification as the negative control. Lanes 3 to 5 are amplified products from DNA of one, three and five nuclei, respectively.

Total barley genomic DNAs were prepared from etiolated young leaves by the standard CTAB procedure. When the total genomic DNA was used as a template, two PCR fragments were amplified (Fig. 1, lane 1). The position of the shorter band is the target product with the expected length of 142 bp, indicating that the design of AMY1 primers is effective for PCR amplification. The longer PCR band of around 350 bp derived from genomic DNA has not been reported previously.

These two PCR products were also effectively amplified from microisolated nuclei. The procedures of microisolation for nuclei are identical with those for chromosomes. When the single nucleus was used as the template, we failed to detect the amplified fragments (Fig. 1, lane 3). We could, however, confirm the two products amplified from more than five nuclei at the identical positions with the genomic DNA template (Fig. 1, lane 5). From these results, we concluded that the five nuclei are sufficient for direct amplification of the target gene. These results also suggest that the isolated nuclei are useful as the template of PCR instead of the genomic DNAs in a similar manner.

#### *Direct amplification of $\alpha$ -amy1 gene from isolated chromosome 6*

To develop a more specified direct amplification technique, we isolated the target chromosome 6 on which the  $\alpha$ -amy1 gene is located, and used them as the template for PCR. The result shows that the expected fragments of 142 bp were amplified by using five microisolated chromosome 6 as the template for PCR (Fig. 2, lane 2). However, we could not confirm the reproducible amplification with one to four chromosome 6. In Fig. 2 (lane 1), the result with two isolated chromosome 6 was shown as an example. Furthermore, no

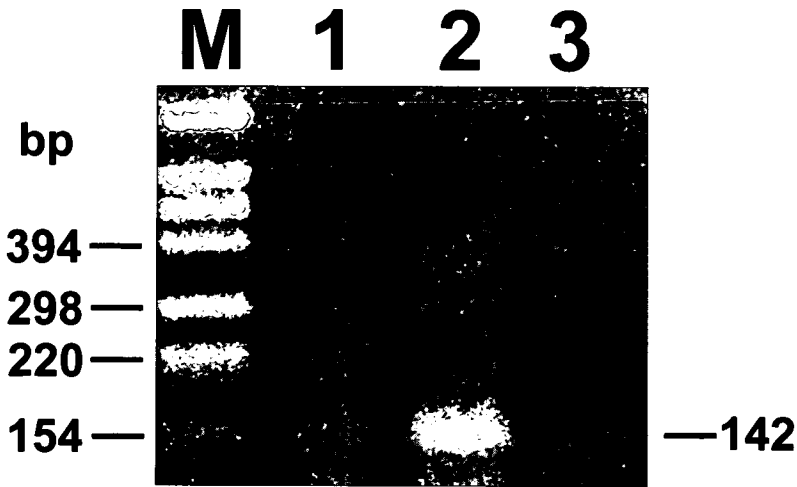


Fig. 2. Agarose gel electrophoresis of PCR products obtained with barley  $\alpha$ -amylase sequence specific primers and template chromosomes. Lane M is DNA molecular marker VI. Lanes 1 to 3 are amplified products from two chromosome 6, five chromosome 6 and five chromosome 7, respectively.

PCR product was detected when five chromosome 7 on which no  $\alpha$ -amylase gene located, were used as a negative control (Fig. 2, lane 3).

From these results, we directly confirmed that  $\alpha$ -*amy1* gene locates on barley chromosome 6, and it can be directly recovered from the isolated chromosome 6. It is also concluded that five isolated chromosomes (or nuclei) are sufficient for direct amplification of the target gene even with the relatively small copy number. Although the method of the direct amplification of a plant gene using laser dissection has been developed, only the multiple copy number genes, such as ribosomal RNA genes (rDNAs) were amplified from the isolated chromosomes and/or nuclei (Fukui et al. 1994, Sawahel and Fukui 1995). We report here the first successful direct amplification of the gene, which have relatively small copy number from isolated chromosomes.

These results also suggested the possibility that the additional 350 bp fragment amplified by PCR with the genomic DNA is located on the another chromosomes other than chromosomes 6 and 7, because no amplified fragment was obtained from these templates (cf. Fig. 1, lane 1; Fig. 2, lanes 2 and 3). The sequences and properties of the amplified fragment with 350 bp are now under investigation.

The direct amplification method is particularly useful for the gene cloning of which physical localization on chromosome is identified by genetic analysis (Macas *et al.* 1993b, Sorokin *et al.* 1994). In addition, the laser dissection can be applied to dissect out specific regions of the chromosome to the size of  $0.5 \mu\text{m}$  with fine laserbeam (Kamisugi and Fukui 1996). This indicates that a combination of direct amplification and laser dissection would greatly facilitate the genome researches. In the case of the physical mapping for example,

genetically determined distances often do not match well with the physical distances (Fukui and Kakeda 1990), however, by using this combination technique, we could define the target gene onto the chromosomal region within 0.5  $\mu\text{m}$  width.

Moreover, we could also dissect out the single C-bands by laser beam (Kamisugi *et al.* 1993). This indicates the feasibility that we could isolate the defined DNA marker for chromosome walking (positional cloning) more easily, even for plant chromosomes without comprehensive genetic maps.

In addition, direct amplification technique can be applied to cloning of chromosome-specific DNA sequences from any plant, regardless of the species, the size of the genome or chromosomes (Kamisugi and Fukui 1996). For example, valuable plant species such as higher plants in fear of extinction, we need only a portion of a root tip for DNA amplification (Nakamura and Fukui 1997).

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