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Fast screening of rice knockout mutants by multi-channel microchip electrophoresis

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ABSTRACT

A multi-channel microchip electrophoresis (MC-ME) system with a laser-induced fluorescence detector was developed for the fast simultaneous detection of rice knockout mutants in genetically modified (GM) rice. In addition, three parallel separation channels were fabricated on a glass microchip to investigate the possibility of high-throughput screening of amplified-polymerase chain reaction products representing wild-type rice and mutants. The MC-ME system was developed to simultaneously record data on all channels using specifically designed electrodes for an even distribution of electric fields, an expanded laser beam for excitation, a 10 × objective lens to capture emissions, and a charge coupled device camera for detection. Under a programmed electric field strength and a sieving gel matrix of 0.7% poly(ethylene oxide) (M_r = 8,000,000), T-DNA-inserted rice mutants, two standard wild-type rice lines, and six rice knockout mutants were analyzed within 4 min using three parallel channels on the microchip. Compared to conventional microchip electrophoresis, the MC-ME method is a valid and practical way to effectively analyze multiple samples in parallel for the identification of GM rice without any loss of resolving power or reproducibility. The MC-ME method was more than 15 times faster than traditional slab gel electrophoresis and proved to be a powerful tool for high-throughput screening of GM rice with high sensitivity, efficiency, and reproducibility.

1. Introduction

More than half of the world's population consumes rice as a staple food [1]. For thousands of years, farmers and later scientists have altered rice to improve characteristics such as grain size, disease resistance, and flavor. The generation of transgenic rice lines for scientific research and commercial applications has been accelerated by the development of technologies for rice transformation and the sequencing of the rice genome [2,3]. Recently, different rice lines have been generated, including mutants lacking genes through knock-outs or with genes newly activated by enhanced gene expression [4]. In addition, foreign genes can be introduced to confer new functions [5]. However, despite the increasing commercial value of genetically modified organisms (GMOs), there is still controversy surrounding the use of genetically modified (GM) crops and foods. Therefore, accurate and fast analytical methods for the identification of GMOs, including GM rice, are required.

Several GM rice assays have been developed, incorporating approaches such as enzyme-linked immunosorbant assay (ELISA), real-time polymerase chain reaction (RT-PCR), quantitative polymerase chain reaction (PCR), and biosensors [6–11]. Among these technologies, slab gel electrophoresis is most commonly used for the detection of amplified DNA fragments related to GM crops and GM foods. However, conventional slab gel electrophoresis for the detection of PCR products has limitations such as a tedious procedure, long analysis time, poor sensitivity, and difficulty identifying heterozygous DNA [12]. In contrast, microchip electrophoresis (ME) has proven to be a compact and attractive format for DNA analysis due to its small sample consumption, short analysis time, high separation efficiency, and high sensitivity [13–17]. ME methods for the assays of GM soybean and GM maize based on single-channel microchips were reported by Kang et al. [18,19]. However, the ability of the ME system to be a practical and highly efficient assay for high-throughput screening remains limited unless more samples can be analyzed in parallel. For the extensive application of GM rice, the time- and cost-savings that a high-throughput DNA analysis method can offer are critical.

The inherent characteristic of microchip devices is their ability to increase throughput by performing parallel analyses of multiple samples on a single microchip. Therefore, the multi-channel microchip electrophoresis (MC-ME) system is a practical and valid way to meet the demand for high-throughput analysis. Microfabricated capillary array electrophoresis coupled with a confocal fluorescence scanning system has demonstrated powerful

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potential in high-throughput analysis [20–24]. The highest throughput system available, with 768 channels on a micro-device, was developed by Aborn et al. [25]. Several improvements have also been explored to improve the performance of multi-channel micro-devices using optically gated injections, acousto-optic deflection-based beam scanning detection, and charge coupled device (CCD)-based transmission imaging spectrographs [26–28]. Simultaneous fluorescence excitation and acquisition systems have been developed based on these technologies. Dang et al. reported a novel multi-channel genetic analysis system based on a poly(methyl methacrylate) plastic chip for high-throughput genetic analysis [29,30]. Shen et al. presented a multi-channel analysis system with a glass microchip for the electrophoretic separation of biological samples [31]. The advantages of these schemes were that separations were detected in parallel without implementing moving parts, and the configurations of channels on the chip did not affect detection.

In the present study, the feasibility of a home-built MC-ME system with improved resolving power and reproducibility using the programmed field strength gradient (PFSG) method is demonstrated for the first time by performing fast simultaneous analysis of GM rice on a three-channel microchip. The MC-ME system was introduced for the rapid simultaneous detection of large numbers of samples and standards (i.e., 50-bp DNA ladder, rice varieties and genotyping of rice knockout mutants) with high efficiency and reproducibility. The PFSG method was also used for faster DNA separation without loss of resolving power.

2. Experimental

2.1. Reagents and materials

A 1 × TBE buffer (0.089 M Tris, 0.089 M borate, 0.002 M EDTA, pH 8.44) was prepared by dissolving a pre-mixed powder (Amresco, Solon, OH, USA) in deionized water. A dynamic coating gel was made with 1.0% (w/v) poly(vinylpyrrolidone) (PVP, M_r=1,000,000) (Polyscience, Warrington, England) in the 1 × TBE buffer containing 0.5 μg/mL ethidium bromide (EtBr, Molecular Probes, Eugene, OR, USA). The mixture was shaken for 5 min and left to stand for 2 h in order to remove bubbles. The sieving matrix was prepared by dissolving 0.7% (w/v) poly(ethylene oxide) (PEO, M_r=8,000,000) (Sigma, St. Louis, MO, USA) in the 1 × TBE buffer with 0.5 μg/mL EtBr by slow stirring overnight. A 50-bp DNA ladder (Carlsbad, CA, USA) was used for electrophoresis and diluted to 25 ng/μL with 1 × TBE buffer before use.

2.2. Rice lines

A knockout mutant for a gene (LOC-Os02g15690) (Fig. 1) putatively encoding pectinase was selected from a rice mutant library that was previously prepared using an *Agrobacterium*-mediated transformation system [32]. Six seeds of the mutant rice line, donated by the Crop Biotech Institute of Kyung Hee University in Korea, were used for this study. Wild-type (WT) rice and mutant rice seeds are shown in Fig. 1(A). Dongjin (DJ) and Hwayoung (HY), two japonica rice varieties, were used as the control lines. Mutant rice was obtained by T-DNA insertion on the rice chromosome Fig. 1(B). Two types of knockout rice were recognized as results of the T-DNA insertion into the rice chromosomes: a homozygous mutant with insertions in both chromosomes (Ho), and a heterozygous mutant with an insertion in one of the chromosomes (He).

2.3. Rice genomic DNA preparation and PCR conditions

For PCR-based genotyping, genomic DNA from each of the rice lines was prepared via the modified hexadecyltrimethylammonium bromide (CTAB) method developed by Rogers and Bendich [33]. Briefly, 0.1 g of powdered rice tissues were suspended in 1 mL of pre-warmed (60 °C) CTAB buffer containing 2% CTAB, 100 mM Tris–HCl, 20 mM EDTA, 1.4 M NaCl, 0.2% (v/v) β-mercaptoethanol, and 0.1 mg/mL proteinase K (pH 8.0), extracted with an equal volume of chloroform:isoamyl alcohol (24:1, v/v) after spin down, and then 0.6 mL of isopropanol was added for DNA precipitation. The DNA pellet was resuspended in TE buffer (100 mM Tris–HCl, 20 mM EDTA, pH 8.0), and 2 μL of the genomic DNA was used for PCR. The PCR reaction was performed in a thermal cycler (Biometra, Germany) according to the following temperature protocol: pre-denaturation at 95 °C for 5 min, followed by 37 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, and a final extension at 72 °C for 7 min. The primer sets included a cultivar-specific primer (marker name RM164, forward, 5′-TCTTGCCCGTCACTGCAGATATCC-3′; reverse, 3′-GCAGCCCTAATGCTAC AATTC TTC-5′) for the identification of rice variety (DJ or HY) based on marker sequences [34]; a vector specific primer (VS) (5′-TTGGGGATCCTCT AGAGTCGAG-3′); and a polysaccharide-degrading enzyme gene (LOC-Os02g15690) primer (PDE) (forward, 5′-TCTAGTTGTTGCATGGTGGT-3′; reverse, 3′-ATGCATGAATTCCAGA AGGT-5′) for genotyping the wild-type and mutant rice. The PCR products were analyzed using slab gel electrophoresis and the MC-ME system.

2.4. Home-built multi-channel microchip electrophoresis system

Fig. 2(A) shows the experimental setup of the MC-ME system. A diode-pumped solid-state (DPSS) laser (model GL-532T-030, λ_{ex}=532 nm, power=30 mW; Shanghai Laser & Optics Century Co., Ltd., Shanghai, China) was used for excitation. A 100 mm focal length cylindrical lens was used to focus the laser beam on the channels of the multi-channel microchip as a line-shape. Only the central part of the laser beam was used, to make the energy distribution as even as possible. A CCD detector (model 01-EXI-BLU-R-F-M-14-C, QImaging Co., Ltd., Surrey, BC, Canada) was used to monitor the fluorescence signal from the channels. A band-pass filter (model 35–5081, 600.0 ± 8.0 nm; Ealing Catalog Inc., Rocklin, CA, USA) was placed in front of the CCD camera to filter out the laser scatter. The laser beam was tilted 45° toward the level to prevent the excitation beam from directly entering the CCD camera, which was investigated with a microscope (model IMT-2, Olympus Co., Tokyo, Japan). An objective lens (10 × /0.25 NA; Olympus Co., Tokyo, Japan) was used to collect the fluorescence levels of the samples in the microchip. The detector was controlled by a PC (3.20 GHz Intel Core i3 PC) with Image-Pro Plus Software (Version 7.0.1.658, Media Cybernetics, Inc., Bethesda, MD, USA). Fig. 2(B) and (C) show the schematic diagram of the high-voltage device (DBHV-100, NanoEntek, Inc., Seoul, Korea) and the specifically designed electrodes (E1, E2, E3, and E4), respectively.

2.5. Multi-channel microchip electrophoresis

The glass multi-channel microchip (NanoEntek, Inc., Seoul, Korea) used in this study had three uniform channels (i, ii, and iii) with 10 reservoirs on the multichannel microchip. The chip channel was 70 μm wide and 10 μm deep, and was connected with reservoirs of 2.0-mm diameter. In each channel of the multi-channel microchip, the injection design was a double-T channel Fig. 2(B). The lengths of the injection channel and the separation channel were 10 mm and 60 mm, respectively. Detection was performed at a distance of 50 mm from the injection position of the double-T channel. The running buffer was 1 × TBE buffer (pH 8.44) with 0.5 μg/mL EtBr. The coating gel and separation sieving gel were 1.0% PVP (M_r=1,000,000) and 0.7% PEO (M_r=8,000,000), respectively, dissolved in 1 × TBE buffer with

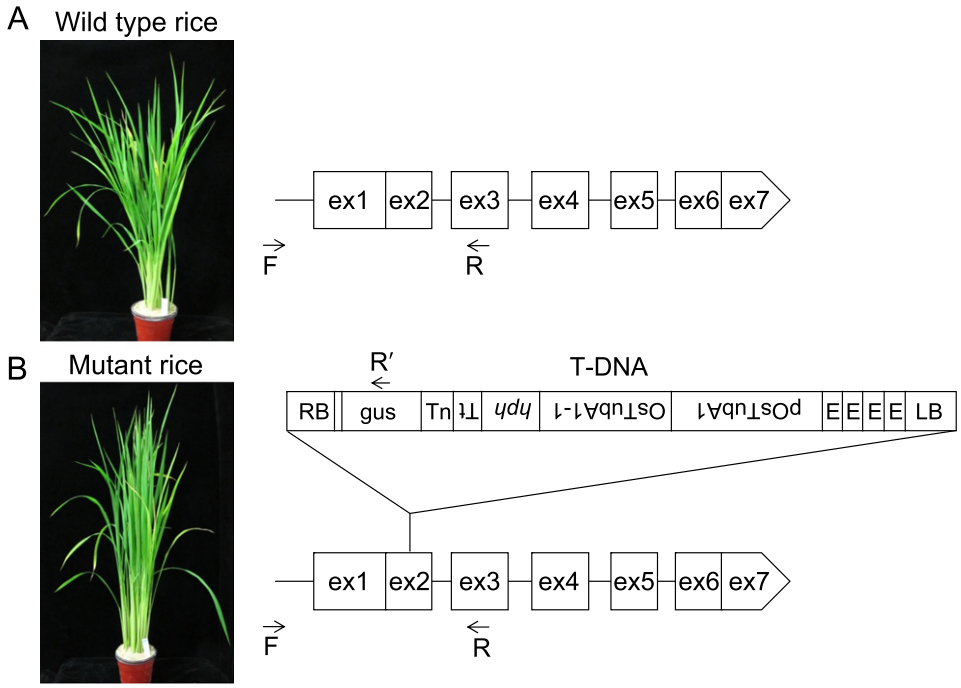

Fig. 1. (A) Photographs of wild-type and mutant rice. (B) Schematic diagram of the wild-type and T-DNA-inserted mutant rice genomes. Indicators: F, forward primer of the cultivar-specific primer; R, reverse primer of the cultivar-specific primer; R′, vector-specific primer; ex, exon; RB and LB in the gray bar represent the right and left borders of the T-DNA, respectively; Gus, GUS reporter gene; Tn, nos terminator; Tt, OsTubA1 terminator; hph, hygromycin phosphotransferase gene; OsTubA1-1, the first intron of OsTubA1; pOsTubA1, the promoter of OsTubA1; E, enhancer element of the CaMV 35S promoter.

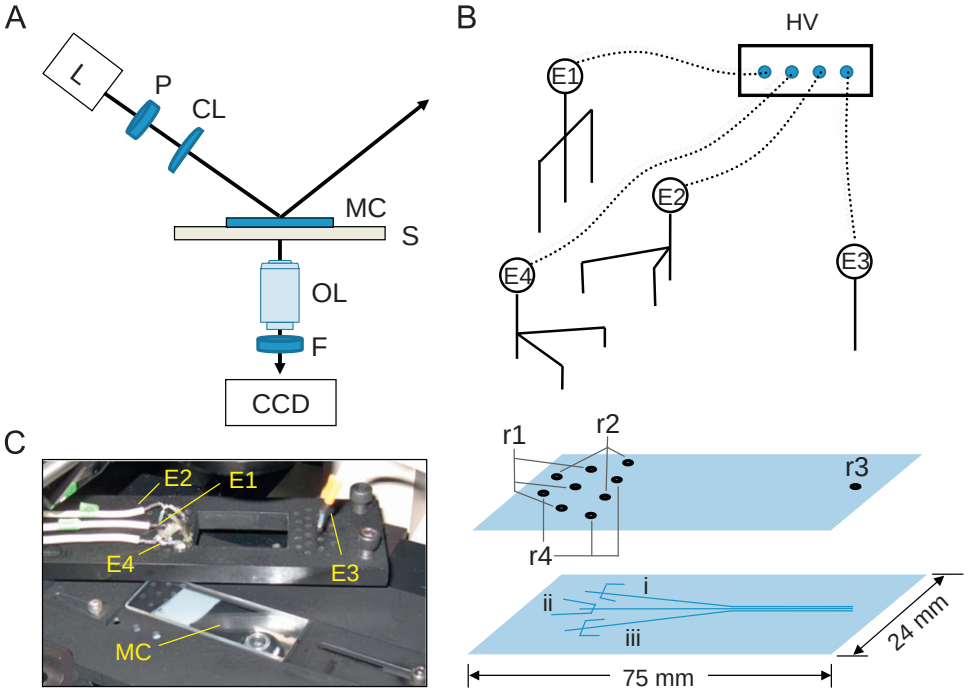

Fig. 2. (A) Schematic diagrams of the MC-ME system based on an inverted type microscope. (B) Design layout of the multi-channel microchip and electrodes. (C) A photograph of the multi-channel microchip and electrodes. Indicators: L, laser; P, pinhole; CL, cylindrical lens; MC, multi-channel microchip; S, stage; F, band-pass filter; OL, objective lens; CCD, charge coupled device; HV, high voltage power supply; E1, electrode 1; E2, electrode 2; E3, electrode 3; E4, electrode 4; i, ii, and iii, three individual separation channels; r1, reservoir of the buffer inlet; r2, reservoir of the sample inlet; r3, common reservoir of the waste buffer and r4, reservoir of the sample outlet.

0.5 μg/mL EtBr. The separation sieving gel was hydrodynamically filled by a vacuum aspirator (model EFOU-KTPM, Hitachi Industrial Equipment Systems Co., Ltd., Tokyo, Japan) into a microchip reservoir 3 (r3 in Fig. 2(B), lower section) for 5 min. The samples were pipetted into the sample inlet reservoirs (r2 in Fig. 2(B), lower section) and then added to the injection region by electrokinetic injection applying a potential of 480 V at the sample outlet reservoirs (r4 in Fig. 2(B), lower section), and then grounding the sample inlet reservoirs for 60 s. The applied electric field ranged from 66.7 V/cm to 600 V/cm at the buffer inlet reservoirs to the

buffer outlet reservoir (r1 and r3 in Fig. 2(B), lower). After each run of the separation, the multi-channel microchip was rinsed with water and running buffer for 10 min.

3. Results and discussion

3.1. Identification of rice varieties and genotyping of rice knockout mutants

To test the reproducibility and accuracy of the MC-ME system, rice varieties were first identified and genotyped by slab gel electrophoresis (SGE). Cultivar-specific primers were used to amplify two short regions with size ranges of 200 bp–250 bp, in the genomes of both varieties, and one of the two DNA fragments exhibited a size difference between DJ and HY. However, the SGE using 1% agarose gel showed a single band of DNA for each sample, as opposed to two bands (Fig. 3(A), upper section). The resolutions of the DNA fragments were improved by using higher gel concentrations (3% agarose gel) of the SGE (data not shown). However, SGE tests using higher gel concentrations are not commonly used for the qualitative identification of GM crops and GM foods due to high viscosity, long analysis time, and poor reproducibility. These results indicate that the SGE method does not distinguish between varieties or identify parental rice varieties of GM rice.

In the genotyping analysis of the DJ mutant lines using a combination of gene-specific primers and a vector-specific primer in the PCR reaction, heterozygous and homozygous rice knockout mutants were identified by the SGE method using 1% agarose gels (Fig. 3(B), upper section). A heterozygous rice line of DJ exhibits two different alleles of the gene putatively encoding pectinase. Therefore, the two bands that were amplified by the gene-specific primers (~710 bp) and a combination of a vector-specific forward primer and the gene-specific reverse primer (~900 bp) were developed as shown for the He mutant line in the upper section of Fig. 3(B). However, the parental rice variety DJ wild-type yielded only one band when the gene-specific primers were used (WT in Fig. 3(B), upper section), and the homozygous rice line had a DNA fragment amplified by the combination of the vector-specific forward primer and the gene-specific reverse primer (Ho in Fig. 3(B), upper section). However, the gene-specific primers used in the PCR process for the heterozygous mutant line demonstrated much lower amplification than the products from the combination of the vector-specific forward primer and the gene-specific reverse primer. We hypothesize that there is competition between the primers and/or different polymerization efficiencies of the unequal lengths of DNA fragments. To avoid missing the detection of small amounts of amplification, a much higher concentration of PCR products was required for exact identification with the SGE protocol. Because of the limited separation efficiency and sensitivity of the SGE method, we introduced a MC-ME system with constant electric field and high revolving power and sensitivity.

The effects of different parameters of MC-ME separation, such as the sieving gel matrix, fluorescence dye, and electric field, were examined. PEO gel was selected as the sieving matrix based on previous studies [15–19]. After comparing the effects of different PEO molecular weights and concentrations on the DNA separations, a 0.7% PEO gel (M_r=8,000,000) was used in further experiments. Electric field strength also plays an important role in DNA analysis. High electric field strength in capillary gel electrophoresis is associated with shorter analysis time and a decrease in separation efficiency. Therefore, an electric field increase from 66.7 V/cm to 266.7 V/cm was examined. The separation electric field strength was determined to be 133.3 V/cm for the variety identification and genotyping of mutant rice. For simultaneous sample introductions in the MC-ME system, specifically designed electrodes (E1–E4) and a high voltage power supply were integrated into the MC-ME

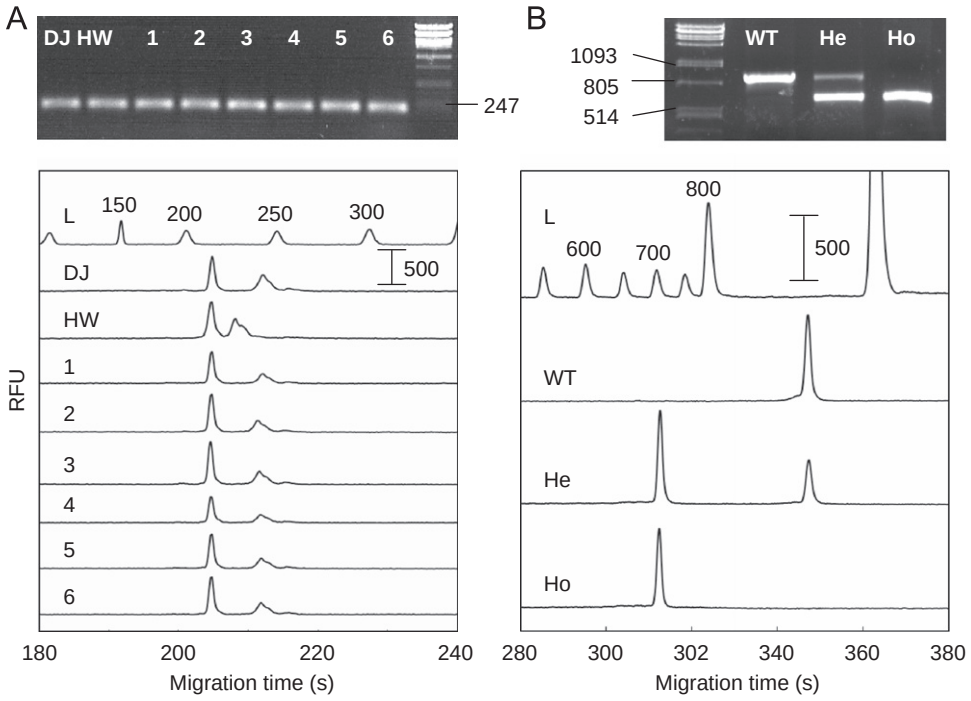

Fig. 3. Representative electropherograms of the SGE (upper) and MC-ME (lower) for (A) the identification of rice species and (B) rice mutant types of DJ. SGE separation conditions: 1% agarose gel matrix in 1 × TBE buffer; applied voltage of 160 V; ambient temperature. ME separation conditions: running buffer, 1 × TBE buffer (pH 8.44) with 0.5 ppm EtBr; coating gel matrix, 1.0% PVP (M_r=1,000,000); sieving gel matrix, 0.7% PEO (M_r=8,000,000); sample injection electric field, 80.0 V/cm for 60 s; applied separation electric field, 133.3 V/cm; microchip effective length, 50 mm. Indicators: L, 50-bp DNA ladder; WT, wild-type; Ho, homozygous mutant line; He, heterozygous mutant line; RFU: relative fluorescence unit.

system. Different samples were simultaneously introduced from sample inlet reservoirs into sample waste reservoirs and analyzed in different channels. A potential of 480 V was applied to the sample waste reservoirs (r4), while the sample inlet reservoirs (r2) were held at ground. The buffer inlet reservoirs (r1) and outlet reservoir (r3) were allowed to float at 60 s. Subsequently, separations were achieved by applying a potential of separation voltage to the buffer outlet reservoir (r3), while the buffer inlet reservoirs (r1) were held at ground.

With constant field strength (CFS), the PCR products amplified by the cultivar-specific primers were analyzed within the MC-ME system. Two wild-type rice varieties (DJ and HY) and six rice mutants (1–6) were clearly differentiated on the electropherogram (Fig. 3(A), lower section). The six rice mutants had the same variety background as the DJ wild-type rice. Compared to the SGE, the separation in the MC-ME had more resolving power and an analysis time that was more than 15 times faster.

A combination of the gene-specific primers and a vector-specific primer were used in the genotyping analysis of the mutant lines. The fast and accurate detection of a heterozygous line and a homozygous line was needed for GM rice research. The lengths of the DNA fragments were determined using a 50-bp DNA size marker. A MC-ME system with CFS was used for the analysis of heterozygous and homozygous mutant lines and revealed a heterozygous rice line (He in Fig. 3(B), lower section) with two different peaks that were amplified by gene-specific primers and by the combination of a vector-specific forward primer and a gene-specific reverse primer, as well as a homozygous rice line (Ho in Fig. 3(B), lower section) with one peak that was amplified by the combination of a vector-specific forward primer and gene-specific reverse primers. Compared to the SGE, the identification of the He and Ho types could be obtained using one-fifth of the volume and a sample concentration diluted 20 times because of the sensitivity of the MC-ME system. These results highlight the potential of the MC-ME system for the fast and sensitive detection of amplified PCR products of rice mutants for variety identification and genotyping.

3.2. Optimization of MC-ME conditions

The PFSG method was introduced to shorten analysis time without any loss of separation efficiency and resolving power. Electric field strength plays an important role in the onset of DNA transport, and therefore separation efficiency [35,36]. According to electrophoretic theory, negatively charged DNA molecules under the influence of an electric field (E) migrate through a buffer with an electrophoretic velocity (V_{EP}), which can be expressed as the product of the electric field and electrophoretic mobility (μ_{EP}) at a given field strength ($V_{EP}=\mu_{EP}\times E$). Since E equals voltage/length, changing the applied voltage is an easy way to control the velocity of the DNA molecules, because it produces variation in different electric fields. Increases in electric field strength increased the velocity of the DNA molecules and reduced migration time. However, higher voltages led to higher currents, causing Joule heating [37]. Since the electrophoretic mobility of DNA molecules depends on electric field strength, the separation time of all DNA fragments can be reduced based on the programmed field strength gradient while maintaining adequate resolution.

Based on simultaneous sample introduction and the detection setups of the MC-ME system, the PFSG method was successfully performed in each channel without any significant crosstalk. Although different electric fields and pulsing parameters were optimal for DNA fragments of different sizes, optimal PFSG conditions were conveniently determined by following normalized strategies that had already been thoroughly studied in our previous papers [15–19]. Furthermore, the PFSG was programmed to yield the best separation of all DNA fragments with resolutions greater than 1.5. Based on the simultaneous sample introduction and detection setups of the MC-ME system, the PFSG method was successfully performed in each channel without any significant crosstalk, as follows. First, to demonstrate the effect of the electric field strength on the resolution under the given conditions, a 50-bp DNA ladder was measured in various electric field strengths from 66.7 V/cm increasing gradually up to 600 V/cm. An increase in electric field strength increased the velocities of the DNA fragments, leading to reduced migration time and resolution. Second, the region of interest on the DNA ladder was determined according to the lengths of target samples. A high electric field strength was applied prior to the first peak and after the last target peak or decrease electric field strength in the region of interest on the basis of the separation efficiency and resolution. Finally, if the second step was acceptable, we tried reducing the gradient time to reduce the run time. At low constant field strength (LCFS, Fig. 4(A)), all of the DNA fragments were separated with adequate resolution by applying a low constant electric field of 66.7 V/cm. However, a total migration time of 762.9 ($\pm$0.7) s was required to separate objective samples. Therefore, the electric field was increased to 600 V/cm (high constant field strength (HCFS), Fig. 4(B)). However, these electric fields were unsuitable for the rapid separation of the target DNA fragments due to the elevated microchip temperature caused by faster axial diffusion from Joule heating. At the same time, the migration times of the DNA fragments were reduced significantly by applying higher electric fields, but the resolution of the smaller DNA fragments was quite poor, with larger DNA fragments overlapping them. Therefore, using an HCFS of 600 V/cm, baseline separation with adequate resolution could not be achieved in a small amount of time. Under these circumstances, the PFSG method (Fig. 4(C)) was required to decrease the migration time and maintain the resolution by changing the electric field during the MC-ME analysis. Using a PFSG of 200 V/cm for 0–185 s, 66.7 V/cm for 185–220 s, 200 V/cm for 220–230 s, 66.7 V/cm for 230–260 s, and

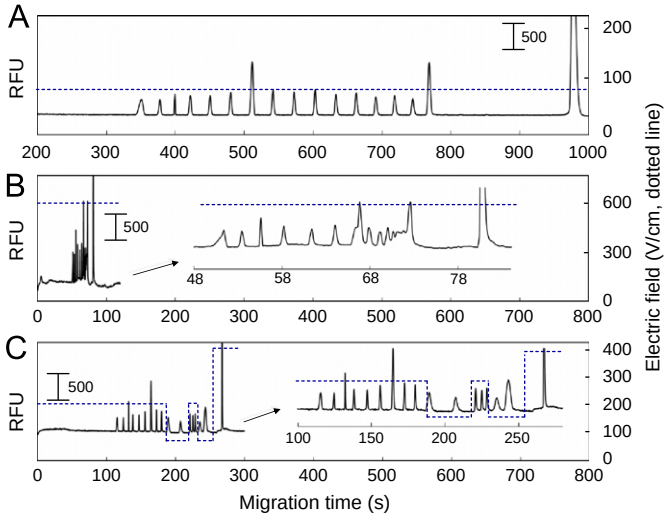

Fig. 4. Representative MC-ME electropherograms of the 50-bp DNA ladder obtained under (A) LCFS, (B) HCFS, and (C) PFSG. ME separation conditions: running buffer, 1 × TBE buffer (pH 8.44) with 0.5 ppm EtBr; coating gel matrix, 1.0% PVP (M_r=1,000,000); sieving gel matrix, 0.7% PEO (M_r=8,000,000); sample injection electric field, 80.0 V/cm for 60 s; microchip effective length, 50 mm. The applied separation electric fields were 66.7 V/cm for LCFS; 600 V/cm for HCFS; and 200 V/cm for 0–185 s, 66.7 V/cm for 185–220 s, 200 V/cm for 220–230 s, 66.7 V/cm for 230–260 s, and 400 V/cm for 260–270 s for the PFSG. RFU: relative fluorescence unit.

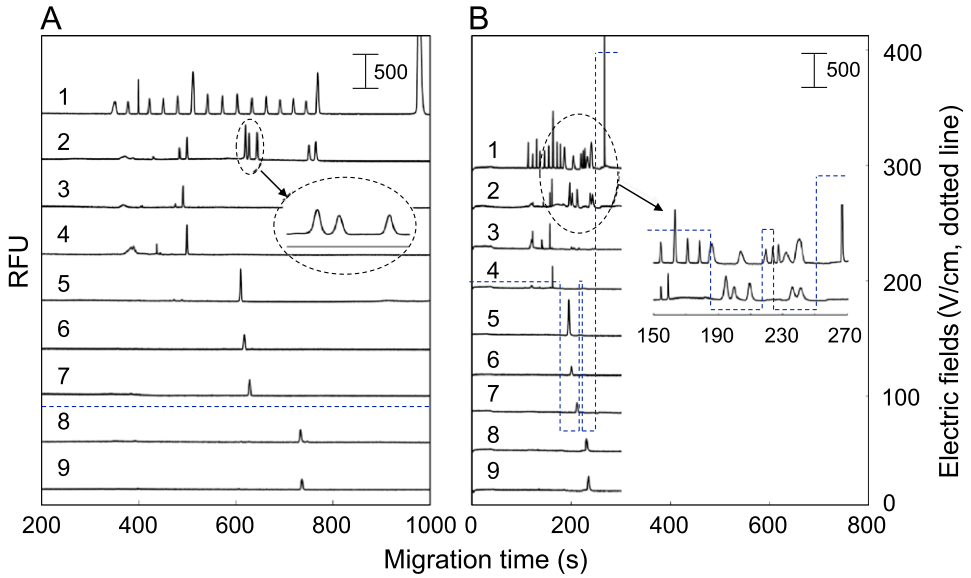

Fig. 5. Representative MC-ME electropherograms of rice knockout DNA samples under (A) CFS and (B) PFSG. MC-ME separation conditions: running buffer 1 × TBE buffer (pH 8.44) with 0.5 ppm EtBr; coating gel matrix, 1.0% PVP (M_r = 1,000,000); sieving gel matrix, 0.7% PEO (M_r = 8,000,000); sample injection electric field, 80.0 V/cm for 60 s. The applied electric field strengths were 66.7 V/cm for CFS; 200 V/cm for 0–185 s, 66.7 V/cm for 185–220 s, 200 V/cm for 220–230 s, 66.7 V/cm for 230–260 s, and 400 V/cm for 260–270 s for PFSG. All other MC-ME conditions are the same as in Fig. 4.

400 V/cm for 260–270 s, the MC-ME method with PFSG was about three times faster than that obtained using the ME-CFS.

3.3. Fast screening of rice mutants by MC-ME with PFSG

Fig. 5 shows representative electropherograms for the PCR products of rice mutants obtained by conventional CFS (Fig. 5(A)) and PFSG (Fig. 5(B)) on a three-channel microchip. The LCFS of 66.7 V/cm was used to determine the separation efficiencies of seven kinds of rice mutants. The resolutions (R_s) of seven DNA fragments were 5.04 (±0.03), 33.88 (±0.97), 1.90 (±0.14), 4.30 (±0.10), 24.95 (±0.10), and 2.77 (±0.11), respectively (Table 1). With the LCFS, all of the DNA fragments were analyzed within 762.9 (±2.3) s (Fig. 5(A)). After applying several PFSGs, however, a suitable PFSG was identified for the rapid analysis of all DNA fragments using an appropriate resolution. Briefly, 200 V/cm for 0–185 s, 66.7 V/cm for 185–220 s, 200 V/cm for 220–230 s, 66.7 V/cm for 230–260 s, and 400 V/cm for 260–270 s was applied for the PFSG. The migration times of all target DNAs were reduced significantly by applying an electric field of 200 V/cm for 185 s (Fig. 5(B)). Under this electric field, the DNA fragments of 250 bp–800 bp were separated with adequate resolution ($R_s > 1.5$). The electric field was decreased in this range, resulting in resolutions of 6.48 (±0.78), 16.69 (±0.95), 1.58 (±0.04), 2.95 (±0.20), 7.18 (±1.67), and 1.52 (±0.01) for seven DNA fragments representing seven rice mutants. The migration times of the seven kinds of DNA fragments were shortened to 156.6 (±0.4), 161.2 (±0.4), 204.6 (±1.4), 210.0 (±1.4), 219.5 (±0.5), 250.8 (±1.1), and 256.1 (±0.9) s, respectively. The reproducibility of the MC-ME with the PFSG method was confirmed by analysis of the 50-bp DNA ladder. Fig. 5 shows the simultaneous determination of seven rice mutants by the MC-ME with a constant electric field and the PFSG method in three channels. Therefore, we concluded that the MC-ME system may be used successfully for the detection of DNA fragments representing rice mutants. Moreover, the PFSG method can be used in the MC-ME system to easily and rapidly evaluate DNA fragment size with high efficiency and low sample consumption. The MC-ME system is expected to be a powerful tool for GM rice research with high resolution and separation efficiency.

Table 1
A comparison of the migration time and peak area of seven rice knockout mutant DNA fragments in a sieving gel containing 0.7% PEO (M_r = 8,000,000), constant field strength (CFS), and programmed field strength gradients (PFSG).

Applied electric field (V/cm)	Rice knockout mutants	Migration time (s)[a]	R_s[b]
CFS	1	485.1 (±2.2)	–
	2	500.5 (±2.0)	5.04 (±0.03)
	3	620.0 (±0.3)	33.88 (±0.97)
	4	627.1 (±0.1)	1.90 (±0.14)
	5	643.3 (±0.5)	4.30 (±0.10)
	6	749.3 (±2.2)	24.95 (±0.10)
	7	762.9 (±2.3)	2.77 (±0.11)
PFSG	1	156.2 (±0.5)	–
	2	160.6 (±0.3)	6.48 (±0.78)
	3	194.4 (±1.7)	16.69 (±0.95)
	4	200.2 (±1.4)	1.58 (±0.04)
	5	209.0 (±0.5)	2.95 (±0.20)
	6	235.8 (±1.1)	7.18 (±1.67)
	7	242.0 (±1.1)	1.52 (±0.01)

[a] Mean ± standard deviation (n = 5).
[b] $R_s = \Delta t / W_{ave}$ (Δt is the difference in the migration time between the two adjacent peaks; W_{ave} is the average peak width of the baseline).

4. Conclusions

A MC-ME system with a laser induced fluorescence detector was investigated for fast GM rice analysis emphasizing high speed, sensitivity, and reproducibility on a multi-channel microchip. Varieties of the rice mutants were identified within only 4 min, more than 15 times more quickly than for traditional slab gel electrophoresis. The MC-ME method also provided fast and accurate identification of rice mutants (i.e., He and Ho) with high sensitivity, which required only one-fifth the volume and 20 times lower concentrations of DNA samples than slab gel electrophoresis. The PFSG method was applied to the MC-ME system for the fast screening of amplified PCR products representing rice knockout mutants. Compared to the conventional ME method, the MC-ME with PFSG on a three-channel microchip yielded sample throughput three times the speed without any loss of resolving power (R_s) or reproducibility. In addition, increases in sample

throughput can be obtained simply by fabricating more channels on the microchip. Each channel was monitored by 8 pixels of CCD camera in the current MC-ME system, and there were 129 pixels in the vertical direction. Considering the spaces between the channels, more than six channels could be etched into the microchips, facilitating even higher throughput analysis.

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