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LI YANG

CHANGCHUN WANG

LIHUAN WANG

CHANGJIE XU

KUNSONG CHEN

TÜBİTAK

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An efficient multiplex PCR assay for early detection of *Agrobacterium tumefaciens* in transgenic plant materials

Li YANG[1,*], Changchun WANG[1], Lihuan WANG[1], Changjie XU[2], Kunsong CHEN[2]

[1]College of Chemistry and Life Sciences, Zhejiang Normal University, Jinhua, Zhejiang 321004, P.R. China

[2]Department of Horticulture, Zhejiang University, Hangzhou 310029, P.R. China

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Abstract: The multiplex polymerase chain reaction (MPCR) is a variant of conventional PCR in which 2 or more target sequences are amplified by more than 1 pair of primers in a single reaction mixture. In this study, an efficient MPCR assay was established and optimized for the quick detection of *Agrobacterium*-derived plant transformants. The neomycin phosphotransferase II gene fragment (*npt*II; target gene) and the chromosomal virulence gene *Chv*A fragment of *Agrobacterium tumefaciens* (external reference gene) were amplified simultaneously with 2 pairs of corresponding primers. The MPCR results showed that 9 out of 10 kanamycin-resistant lines tested were transformed with the foreign genes. This optimized MPCR assay, rapid, sensitive, and reliable, can facilitate the identification of *Agrobacterium*-derived plant transformants at the early stage of selection.

Key words: *Agrobacterium tumefaciens*, multiplex polymerase chain reaction, transgenic plant detection

1. Introduction

Since the first commercial release of genetically modified (GM) tomato in 1994 (Nap et al. 2003), the worldwide acreage devoted to GM crops has increased rapidly to an estimated 160 million ha in 2011. The main GM crops grown commercially are cotton, soybean, maize, oilseed rape, and potato, and many of these crops possess significant advantages over wild-type strains, including increased pest resistance, better flavor, and higher nutrition (James 2011).

GM crops have developed swiftly owing to the application of new techniques that transfer foreign genes into recipient plant cells. The most widely used technique for higher plant transformation is mediated by *Agrobacterium tumefaciens*, a soil plant-pathogenic bacterium (Gelvin 2003). The transformation efficiencies of most plant species are generally low, because most of the buds are nontransgenic escapes. Therefore, a rapid, accurate, and reliable method is required to identify transformed plants at the early stage, in order to replace the conventional expensive and time-consuming procedures.

The traditional method for analyzing the stable genomic integration of a transferred gene is Southern blotting (Southern 1975). However, Southern blotting is complex and time-consuming; moreover, this technique requires at least 10 µg of high-quality genomic DNA. Alternative techniques like plasmid rescue (Grant et al. 1990), inverse polymerase chain reaction (IPCR; Does et al. 1991), random-primed PCR (Swensen 1996), and plant transfer DNA (T-DNA) junction-sequence analyses (Zhou et al. 1997) have also been established to identify the border junction sequences between the T-DNA and the plant genomic DNA to clearly show the integration of the foreign gene.

Of all these detection techniques, PCR is the most commonly applied method for the analysis of genes in tiny samples of DNA because it is highly specific, sensitive, and amenable to full automation (Klapper et al. 1998). To increase the specificity, efficiency, and fidelity of PCR, a variant termed multiplex PCR (MPCR) has been developed (Chamberlain et al. 1988). MPCR is a modified conventional PCR, in which 2 or more primer pairs are included in 1 sample PCR reaction mixture, leading to amplification of multiple products simultaneously. Because it is relatively quick, easy-handling, and cost-effective, in addition to being sensitive and reliable, MPCR has been widely used in iatrology, microbiology, and botany for the diagnosis of mutations and research on functional genes and quantitative trait loci (Elnifro 2000). Permingeat et al. (2002) and James et al. (2003) devised an MPCR protocol for the detection of transgenic materials using primers that target sequences within the functional transgenes, and/or

* Correspondence: yangli@zjnu.cn

the promoter or terminator sequences of the expression vector. However, the method still generates false positives because antibiotics do not always eliminate the residual *Agrobacterium* (Cubero et al. 1999, 2002).

This article describes a rapid DNA-extraction method and an MPCR amplification protocol for the reliable, sensitive, and specific detection of transformed kumquats at the early stage of transformant selection. To this end, 2 pairs of primers were designed to amplify the *Agrobacterium* chromosomal virulence (*Chv*) gene *Chv*A and the kanamycin-resistance gene *neomycin phosphotransferase* II (*npt*II, present in some of the GM products commercially on the market) of the genomic DNA samples obtained from transgenic kumquats with an antisense carotenoid biosynthesis gene beta-carotene hydroxylase.

2. Materials and methods

2.1. Plant materials

Kanamycin-resistant shoots or 3-month-old plantlets of transgenic kumquats (*Fortunella crassifolia* Swingle), transformed with the binary expression vector pBI-aCSBCH (Yang et al. 2007), were used as the source of tissue for detecting the *Chv*A and *npt*II genes. The control nontransformed plants were grown from commercial seeds.

2.2. Genomic DNA extraction

Genomic DNA was extracted from 20–50 mg of young leaf tissue according to the method described by Vickers et al. (1996) with some modifications. Briefly, the leaf segments were ground to a fine powder in 1.5-mL microcentrifuge tubes immersed in liquid nitrogen; the powder was then mixed with 0.6 mL of prewarmed (65 °C) extraction buffer (containing 100 mM Tris-HCl, 40 mM EDTA, and 1% sodium dodecyl sulfate, pH 9.0) and incubated at 65 °C for approximately 30 min. After cooling to room temperature, 0.6 mL of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) was added and the tube was centrifuged at 11,180 × *g* for 10 min at room temperature. The upper phase was extracted once or twice with chloroform/isoamyl alcohol (24:1, v/v), then precipitated by adding a 1/10 volume of sodium acetate (3 M, pH 5.2) followed by 1 volume of isopropanol. The resultant solution was mixed, left undisturbed for 10 min, and then centrifuged at 11,180 × *g* for 5–10 min. The DNA pellet was washed with 70% (v/v) ethanol, air-dried, and suspended in 15 µL of a solution of tris(hydroxymethyl)aminomethane (Tris) and ethylene diamine tetraacetic acid (EDTA). The quality of genomic DNA was assayed by visualization of an aliquot from each sample on an ethidium bromide-stained agarose gel and compared with λ DNA (500 ng μL^{-1}, TaKaRa, Japan). DNA yield was quantified spectrophotometrically. All the DNA samples were then resuspended to yield aliquots of 100 ng μL^{-1} for MPCR analysis.

2.3. Oligonucleotide primers

Two pairs of primers were used in this study. The primer pair of KUP (5′-TGCTCGACGTTGTCACTGAAGC-3′) and KDP (5′-AGCAGGCATCGCCATGGGTCAC-3′) was designed to amplify a 339-bp fragment of the selective marker gene *npt*II (GenBank accession number V00618). The primer pair of ChvAUP (5′-CGAAACGCTGTTCGGCCTGTGG-3′) and ChvADP (5′-GTTCAGCAGGCCGGCATCCTGG-3′) was used to amplify an 898-bp specific fragment of the *Agrobacterium* chromosomal virulence gene *Chv*A (GenBank accession number M24198).

2.4. MPCR conditions

To find the optimal PCR conditions, various PCR parameters were assessed including annealing temperature, relative concentrations of primers, and PCR buffer components. Multiplex PCR reactions were carried out in 0.2-mL tubes in a thermal cycler (Hybaid PCR Express Thermal Cycler, United Kingdom) of the *npt*II and *Chv*A gene fragments simultaneously. Each reaction mixture contained 1 µL of the template DNA (100 ng), 1.5X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM KUP, 0.2 µM KDP, 0.2 µM ChvAUP, 0.2 µM ChvADP, 1 U Taq polymerase (New England Biolabs RTM. Inc, USA), and sterile water to a final volume of 25 µL. Each amplification was repeated at least twice. For optimizing the MPCR performance, volumes of specific reagents were varied as described in the Results section.

Samples were subjected to PCR amplification using a "hot start" PCR program. The program included an initial denaturing step (94 °C for 5 min) followed by 40 cycles of denaturation at 94 °C for 40 s, annealing at 66 °C for 40 s, and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The annealing and extension temperatures were varied while carrying out the optimization. Next, 10 µL of the PCR products were analyzed on 2.0% agarose gels containing 0.5 µg mL^{-1} ethidium bromide in 1X Tris-acetate-EDTA buffer at room temperature using a voltage gradient of 10 V cm^{-1}.

To optimize the annealing temperature (Tm), the theoretical Tms of the primer–template pairs were calculated using the formula Tm = 81.5 °C + 16.6 (log [K+]) + 0.41 (% of [G + C]) – (675/n), as according to Sambrook et al. (2001). The theoretical Tm values of the 4 primers were 68 °C for KUP, 72 °C for KDP, 72 °C for ChvAUP, and 74 °C for ChvADP. Thus, the optimal Tm for the mixture was assayed by using an automated PCR gradient ranging from 50 °C to 70 °C. In the MPCR reaction, 1 primer pair was held at 0.2 mM, while the other was varied to attain ratios of *npt*II and *Chv*A primer concentration between 8:1 and 1:8. To optimize the buffer concentration, the concentrations of $MgCl_2$ and dNTPs were maintained at 2.5 mM and 0.2 mM, respectively, and the buffer was

added at concentrations of 0.5-, 1.0-, 1.5-, 2.0-, and 2.5-fold in the 25-μL reaction volume.

In order to determine the MPCR sensitivity analysis, DNA from a 10-fold dilution series of *Agrobacterium* cells (from 1 up to 10^5) containing the vector pBI-aCSBCH was added to 100 ng of the nontransformed genomic DNA, to ascertain the minimum number of detectable *Agrobacterium* cells in a single reaction and to evaluate the *Agrobacterium* contamination in the resistant shoots or plantlets.

3. Results

3.1. Optimization of multiplex PCR

The presence of more than one primer pair in the MPCR increases the chance of spurious amplification products by the formation of primer dimers. Thus, special attention should be paid in primer design (Elnifro et al. 2000), particularly to primer length, GC content, Tm, and sequences complementary to other targets. It is essential to ascertain the Tm of every primer and to test primer–primer interactions for the specificity and efficiency of PCR. In practice, the Tm is influenced by the individual buffer components, and by both the concentrations and quality of the primers and the template (Roux 1995). In this study, a successful MPCR was obtained by gradient PCR when the annealing temperature was maintained between 50 °C and 70.5 °C. As the temperature increased, spurious amplification was reduced, and the optimal temperature was 66 °C when the concentrations of 2 products were almost equal in proportion (Figure 1).

The relative concentration of the 2 primers pairs is the most critical parameter in MPCR. In these experiments, the optimized concentrations of the primer sets were 0.2 μM for ChvAUP/ChvADP and 0.2 μM for KUP/KDP; moreover, increasing the *Chv*A-primer concentration does not improve the amplification efficiency of the *Chv*A fragment. However, using this MPCR protocol with kanamycin-resistant samples infected with *Agrobacterium*, it was still easy to obtain false-positive results if the concentrations of the 4 primers were equal because amplification of the *Chv*A fragment was prone to interfere with that of the *npt*II fragment, especially when the concentration of *Agrobacterium* was relatively low (data not shown). Edward and Gibbs (1994) proposed that if equimolar primer concentrations did not yield uniform amplification signals for all the fragments, the amounts of some primer pairs should be reduced or increased in relation to others. The recommended final primer concentration for low copy number or high-complexity DNA is 0.3–0.5 μM, whereas for high copy number or low-complexity DNA, the recommended primer concentration is 0.04–0.4 μM (Markoulatos et al. 2002). Thus, maintaining the concentrations of ChvAUP and ChvADP constant at 0.2 μM, the concentrations of the *npt*II primers were diluted by 2, 4, 8, and 32 times, respectively. The optimal concentrations of primers were determined as follows: ChvAUP and ChvADP at 0.2 μM, with KUP and KDP at 0.05 μM (Figure 2).

The standard 10X buffer for conventional PCR contains 10 mM Tris (pH 8.0), 25 mM $MgCl_2$, and 50 mM KCl. Varying the concentrations of the various buffer components can affect amplification efficiency dramatically (Cha and Thilly 1993). Generally, increasing the buffer concentration may improve the efficiency of the multiplex reaction. Moreover, primer pairs generating long amplification products work better at lower salt concentrations, whereas primer pairs producing short amplification products work better at higher salt concentrations (Henegariu et al. 1997). As the stringency in the reaction mixture increases, the longer products of the *Chv*A gene fragment are amplified more efficiently, while the intensity of the *npt*II fragments gradually decreases. In this particular primer mixture, the optimal buffer concentration was determined to be 1.5X standard buffer concentration (Figure 3).

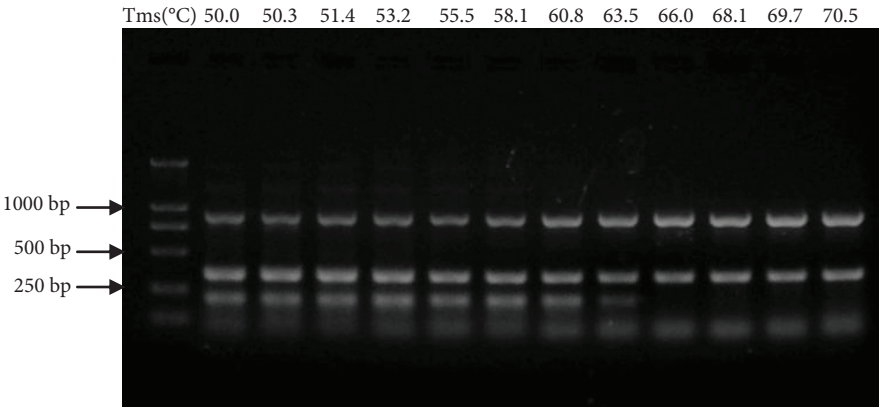

Figure 1. Effects of various annealing temperatures (Tms) on MPCR amplification. The Tms are indicated at the top of each lane. Lane 1 is DL2000 DNA Marker (TaKaRa, Japan).

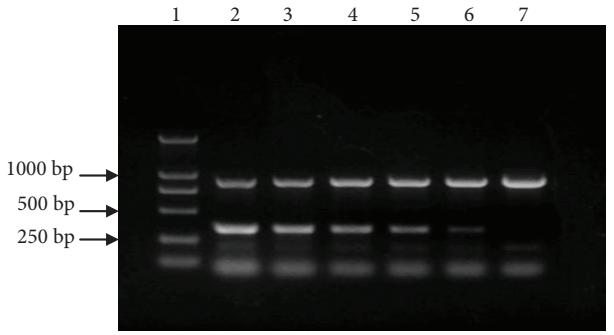

Figure 2. Effects of various primer concentration ratios on MPCR amplification. Lane 1: DL2000 DNA Marker (TaKaRa, Japan); lanes 2–7: 0.2, 0.1, 0.05, 0.025, 0.0125, and 0.0625 μM KUP and KDP (*npt*II-gene primers). The concentrations of ChvAUP and ChvADP (*Chv*A-gene primers) were maintained at 0.2 μM.

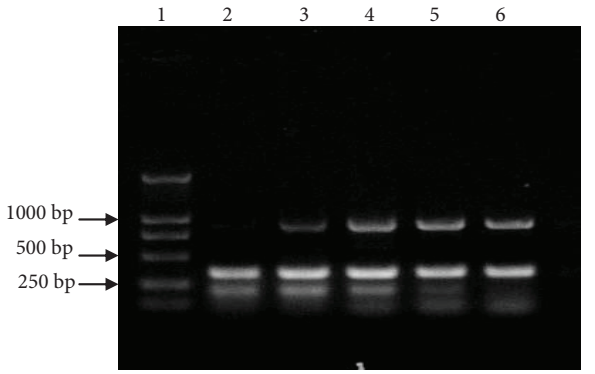

Figure 3. Effects of MPCR buffer concentration on amplification. Lane 1: DL2000 DNA Marker (TaKaRa, Japan); lanes 2–6: 0.5-, 1.0-, 1.5-, 2.0-, and 2.5-fold standard PCR buffer concentration, respectively.

3.2. MPCR sensitivity analysis by detecting *Agrobacterium* cell number

Mogilner et al. (1993) reported that "agroinfected" plants placed in selective media for 38–90 days after inoculation still contained large amounts of bacteria. The same phenomenon was found in our experiments. *Agrobacterium* contamination in the explants was undesirable during the selection procedure because we could not determine whether the "agroinfected" plants were successfully transformed with foreign genes or were still contaminated by *Agrobacterium*. Thus, in order to analyze the sensitivity of the optimized MPCR assay in detecting the level of contamination of *Agrobacterium* in infected transgenic samples, the *npt*II and *Chv*A gene fragments were amplified from 100 ng of nontransgenic plant genomic DNA spiked with DNA derived from 1 to 10^5 cells of *Agrobacterium* (Figure 4). No clear or faint bands were detected when low dilution titers of *Agrobacterium* were tested; however, amplification products of the *npt*II and *Chv*A genes were observed when the cell number reached 10^2 to 10^5.

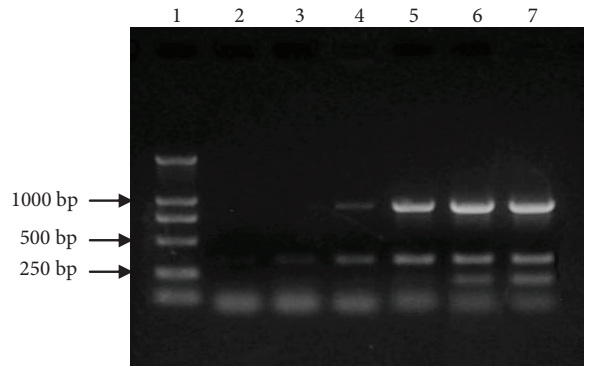

Figure 4. Effects of *Agrobacterium* cell number on MPCR amplification. Lane 1: DL2000 DNA Marker (TaKaRa, Japan); lanes 2–7: 1, 10, 10^2, 10^3, 10^4, and 10^5 *A. tumefaciens* cells containing 100 ng of genomic DNA of nontransgenic plants.

3.3. Application of the optimized MPCR for detecting kanamycin-resistant kumquats

Kanamycin-resistant kumquat shoots, 3-month-old seedlings grafted on parental stock, and nontransgenic plants growing in a greenhouse were used as test samples to evaluate the detection of *Chv*A and *npt*II fragments with the optimized MPCR method (Figures 5a and 5b). Figure 5a shows all the PCR products from resistant shoots amplified by the *npt*II primer set (lanes 2 to 11). Five samples (4, 5, 6, 9, and 11) showed the *Chv*A gene fragment, suggesting that these 5 seedlings were contaminated by *A. tumefaciens*. Compared to the MPCR products of nontransgenic materials containing 10^2, 10^3, and 10^4 *Agrobacterium* cells, we determined that the kanamycin-resistant shoots of lanes 2, 3, 7, 8, and 10 were successfully transformed with the foreign genes (*npt*II gene fragments were amplified alone), while the shoots of lanes 4, 5, and 11 were transformed but were contaminated with *A. tumefaciens* (*Chv*A gene fragments were faint compared with *npt*II gene fragments), and those of lanes 6 and 9 required longer cultivation for Southern blot evaluation, because the *Chv*A gene fragments were amplified strongly compared with *npt*II gene fragments (Figures 5a and 5b). Seven out of 10 kanamycin-resistant shoots grown from *Agrobacterium*-infected explants were successfully detected in this work.

4. Discussion

Agrobacterium-mediated transformation is a major tool for plant molecular breeding. In spite of the high level of efficiency of *Agrobacterium*-mediated gene transformation, the escape of regenerated shoots from the

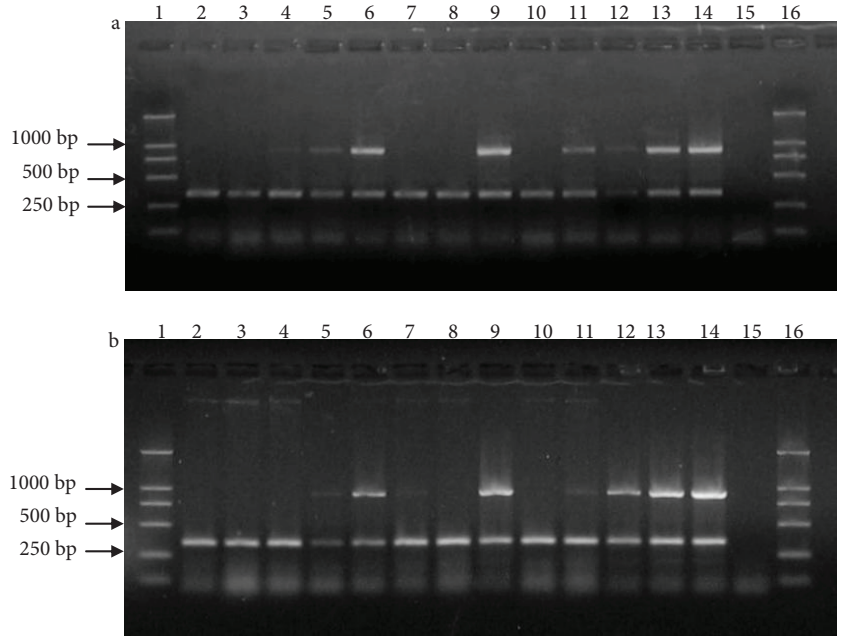

Figure 5. MPCR assay of kanamycin-resistant plant materials. Lanes 1 and 16: DL2000 DNA Marker (TaKaRa, Japan); lanes 2–11: 100 ng of genomic DNA of different kanamycin-resistant lines; lanes 12–14: 100 ng of genomic DNA from nontransgenic kumquat containing 10^2, 10^3 and 10^4 *A. tumefaciens* cells; lane 15: 100 ng of genomic DNAs of nontransgenic kumquat. a) MPCR assay for 3- to 4-month-old resistant shoots regenerated after kanamycin selection. b) MPCR assay for resistant plantlets after grafting 3 to 4 months.

selection medium is still a serious problem. Furthermore, the persistence of *Agrobacterium* after transformation interferes with the identification of transformants and with the growth and development of the transformed plant cells. Indeed, the use of high concentrations of antibiotics could affect plant cells' vitality, killing transformed plants because antibiotic efficacy is limited (Tang et al. 2000). Thus, it is necessary to identify the transformed shoots at the early stage of transformation to save labor and time.

During *Agrobacterium*-mediated genetic transformation, a number of tumor-inducing plasmids and chromosomal virulence genes for insertion of foreign genes into the plant genome are required (Gelvin 2003). The chromosomal virulence loci *Chv*A and *Chv*B are required for attachment of bacteria to the plant cell (Garfinkel and Nester 1980; Douglas et al. 1985). The *Chv*A gene is indispensable for the transport of a small polysaccharide, β-1,2-glycan, from the bacterial cell into the plant cells, an early event in crown-gall tumor formation. Residual *A. tumefaciens* in plant materials that have been infected can be easily diagnosed by amplifying the *Chv*A gene fragment. The *npt*II gene is a common selection marker for plant expression vectors and so is often used for the detection of GM plants. By using these 2 genes as target genes, a simple and efficient MPCR method was developed to distinguish transformed plants and wild-type plants and to detect the residual contamination from *Agrobacterium* in plants at an early stage of transformation.

MPCR is a process that simultaneously amplifies multiple sequences in a single reaction. It is fast becoming a common screening assay in both clinical and basic research (Elnifro 2000). However, many factors can affect the specificity, efficiency, and sensitivity of amplification. For a successful MPCR, the relative concentrations of the primers, PCR buffer and dNTPs concentrations, cycling temperatures, and the amounts of template DNA and Taq DNA polymerase should be thoroughly evaluated (Cha and Thilly 1993; Edwards and Gibbs 1994; Brownie et al. 1997; Elnifro et al. 2000; Markoulatos et al. 2002). Our results demonstrated that annealing temperature, relative concentrations of the primers, and PCR buffer concentration were the chief parameters that influenced MPCR and need to be optimized to obtain an efficient MPCR assay.

In summary, an efficient, reliable, and sensitive MPCR system has been developed to detect transformed seedlings at an early stage after transformation. Transformed samples, transformed samples contaminated with *Agrobacterium*, and nontransformed samples can be distinguished using the dual primer set *npt*II/*Chv*A, while an engineered *Agrobacterium* containing the binary

expression vector can be used as a positive control. Using the optimized MPCR, 7 out of 10 *Agrobacterium*-derived plant transformants were successfully detected at the early stage of selection in this study. Thus, this protocol should be useful for the high output detection of transformed buds or shoots in the early transformation stage.

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