Detection of the CaMV-35S Promoter Sequence in Maize Pollen and Seed

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ABSTRACT

This study developed an extraction protocol for pollen DNA in corn, and screened current and new primers designed to detect the CaMV35S promoter in corn pollen and seed. Bt transgenic and non-transgenic corn hybrids were used to obtain the seed and pollen DNA. Polymerase Chain Reaction (PCR) and gel electrophoresis were used to evaluate the efficacy of the pollen DNA extraction protocol, and to test the efficiency of 11 primer pairs in detecting the CaMV35S promoter sequence. The DNA extraction method described here was very successful in releasing the DNA from pollen grains, as determined by the intensity of the 18 bands of genomic DNA samples amplified with the HMG-AF1/HMG-AR1 corn-specific primers. The strong intensity of the bands formed by primers P35S1/P35S2, P35SA/P35SB and P35S-aflu/P35S-arl showed these primers were the most efficient in amplifying transgenic pollen DNA; whereas, primers P35S1/P35S2 generated the strongest band intensity in seed DNA. The new primers 35S168F/35S317R showed higher sensitivity in detecting the CaMV35S promoter than any other primer included in this experiment. The proposed pollen DNA extraction method and the primer 35S168F/35S317R were very effective in extracting DNA from pollen samples and identifying the CaMV535S promoter sequence in transgenic varieties.

INTRODUCTION

Several crops have been improved by the introduction of novel genes. These genes are inserted using genetic engineering techniques. They can be cloned from a related or unrelated species (Potrykus, 1991). Some examples are traits for tolerance to herbicide and insect resistance. More recently, transgenic crops have been transformed to produce plant-made vaccines and therapeutic proteins. While the new crops represent many advantages to crop productivity and profitability, many scientists have expressed concern about the potential movement of these genes into conventional crops by pollen flow and their possible impact on the environment (Dale et al., 2002; Nordlee et al., 1996; Quist and Chapela, 2001). Testing of conventional seed and grain for the presence of adventitious biotech traits is mandatory in the European Community (European Commission, 2004). Detection of transgenes can be accomplished by DNA, RNA, or protein isolation techniques. DNA methods are preferred because of stability of DNA during extraction, purification, PCR amplification, sensitivity and capability to detect a wider range of constructs (Giovanini and Concilio, 2002).

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Holst-Jensen et al. (2003) have classified transgenic DNA detection methods into four categories: 1) screening for 35S and NOS specific sequences that can detect a wide range of transgenic traits; 2) gene specific detection for identification of a transgene; 3) construct specific detection for the identification of a gene construct used in the transformation process; and 4) event specific detection methods that identify the insertion site of the transgenic trait in the genome. Some examples of the use of these methods are detection of the CaMV-35S promoter (Lipp et al., 2001), identification of the bar gene (Kota et al., 1999) and CryIAb gene (Studer et al., 1998), junction identification of the P-35S-heat shock protein 70 intron I in Mon810 maize (Zimmermann et al., 1998), and junction detection of the host plant genome-integrated recombinant DNA in Bt11 maize (Zimmermann et al., 2000).

Several studies have been conducted to develop a 35S promoter specific screening method for biotech traits using DNA from grains or processed food (Lipp et al., 2001; Tozzini et al., 2000; Vollenhoffer et al., 1999). Some of the genetic markers cited in the literature for the identification of transgenic events in corn are considered unreliable and result in a high number of false positives and negatives during PCR amplification (Christou, 2002). Although there is a reliable protocol for the extraction of DNA from single corn seed (Sangtong et al., 2001), there is little published information on the extraction of DNA from pollen samples. Therefore, the objectives of this study are to develop a protocol to extract DNA from corn pollen, to screen molecular markers available in the literature, to develop new markers for detecting the CaMV-35S promoter in transgenic corn pollen and seed, and to design new primers from the 35S sequence and compare them to other published studies.

MATERIALS AND METHODS

Plant material and DNA extraction and quantification.

Seed DNA extraction. Ten mg of corn meal from transgenic hybrids of FR1064×LH185Bt, LH245×LH185Bt, SGI928×HC50Bt, HCSOBtxSGI905, TR7322×MBS1236Bt, DKC69-71, 4-NK7070Bt, and Asgrow RX792 (non-transgenic hybrid) were used to extract DNA by the extraction method described by Sangtong et al. (2001). Corn meal was extracted from the endosperm of 10 seeds of each hybrid, using a Craftsman rotary drill. Plant Tissue Protocol from PUREGENE Genomic DNA Purification Kit of Gentra Systems (Minneapolis, MN) was used to extract DNA from the corn meal. The procedure followed for the extraction of DNA from the samples is described in the PUREGENE kit. An abbreviated description of the DNA extraction steps includes adding 300 µl of Cell Lysis Solution to the corn meal and mixing by vortexing (Genie 2™, Fisher Scientific, Bihema, NY) the tube for 3 s. Cell lysate was incubated (Dry bath incubator, Boeckel Scientific, PA) at 65 °C for 60 min. After incubation, 1.5 µl of RNAase A solution was added to the cell lysate. Next, samples were incubated in an oven (Thelco, GCA/Precision Scientific, Chicago, IL) at 37 °C for 40 min. After cooling the samples to room temperature, 100 µl of the Protein Precipitation Solution were added to the cell lysate. The solution was mixed by inverting the tube several times and then incubated for 20 min

in an ice bath. The protein precipitation solution was mixed by vortexing the tube for 20 s. Next, the tube was centrifuged (Centrifuge 5417C, Eppendorf, Westbury, NY) at 14,000 rpm for 3 min. The supernatant containing the DNA was transferred to a clean 1.5 ml centrifuge tube containing 300 µl 100% isopropanol. After mixing gently by inverting the tube several times, the tube was centrifuged at 14,000 rpm for 2 min. The supernatant was poured off and the tube was drained on clean absorbent paper. The DNA pellet remained in the tube and was washed by adding 300 µl of 70% ethanol and gently mixing. Next, the tube was centrifuged at 14,000 rpm for 2 min and the ethanol was poured off. The tube with the DNA pellet was inverted and drained on clean absorbent paper and air-dried for 15 min. The DNA was rehydrated by adding 50 µl of DNA hydration solution and incubating the sample for 1 h at 65 °C.

Pollen DNA extraction. Pollen DNA was extracted from three corn varieties: Asgrow RX792 (a non-transgenic hybrid), DKC69-71 (a transgenic hybrid), and an experimental HiII transgenic corn variety provided by Dr. Kan Wang, Iowa State University Plant Transformation Facility (Armstrong et al., 1991). The commercial and the experimental hybrids were grown in the field and a greenhouse, respectively. DNA was extracted using a modified pollen DNA extraction protocol provided by Dr. Paul Scott, USDA-ARS at Iowa State University. Some additional steps were added, following the recommendations in the PUREGENE DNA purification kit. Pollen was collected from the field and greenhouse-grown corn plants. Field samples were collected into a 50 ml centrifuge tube taped to a plastic funnel of 18 cm in diameter. The funnels were supported with wire to a garden stake and placed at the height equivalent to the ear in the corn plant. Tubes were replaced daily. Collected samples were immediately frozen at −80 °C until used for DNA extraction.

An alternative procedure was used to break the pollen grains and release the DNA. The DNA extraction procedure consisted of mixing 10 mg of pollen grains with 400 µl of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Two steel beads were added to grind the pollen grains. The tubes were shaken for one minute with an experimental DNA sample grinder developed by Drs. Yuh-Yuan Shyy and Rai Satish at the Seed Science Center, Iowa State University. The cell lysate was transferred to a new tube for incubation at 65 °C for 60 min. After incubation, the samples were cooled at room temperature and mixed with 400 µl of saturated phenol. Next, the tubes were placed on ice for 30 min and then centrifuged for 10 min at 12000 rpm. The aqueous phase was pipetted into a clean 1.5 ml centrifuge tube and mixed with 400 µl of chloroform. The tubes were centrifuged for 10 min at 12000 rpm. Then, the aqueous phase was pipetted into a clean 1.5 ml centrifuge tube and mixed with 400 µl of ice-cold isopropanol. The tubes were left on a bench at room temperature for 15 min and then centrifuged for 10 min at 14000 rpm. The isopropanol was discarded and the tube was inverted and drained on clean absorbent paper. The DNA pellet was washed with 300 µl of 70% ethanol. The tube was centrifuged for 2 min at 14000 rpm, the ethanol was poured off, the tube was inverted and drained on clean absorbent paper, and the samples air-dried for 10 min. The DNA pellet

TABLE 1. Name, sequence, and amplicon length of primers.

Primer Num.	Name	Sense sequence Antisense sequence	Amplicon size (bp)	Reference
1	P35S-1 P35S-2	5'-GCTCCTACAAATGCCATCA-3' 5'-GATAGTGGGATTGTGCGTCA-3'	195	Lipp et al., 1999; Tozzini et al., 2000; Windels et al., 2001
2	P35S-A P35S-B	5'-AAGGGTCTTGCGAAGGATAG-3 5'-AGTGGAAAAGGAAGGTGGCT-3'	226	Lipp et al., 2001
3	P35S-cf3 P35S-cr4	5'-CCACGTCTTCAAAGCAAGTGG-3' 5'TCCTCTCCAAATGAAATGAACTTCC-3'	123	Lipp et al., 2001
4	P35S-aflu P35S-ar1	5'-CCTACAAATGCCATCATTGCG-3' 5'-GGGTCTTGCGAAGGATAGTG-3'	205	Pietsch et al., 1997; Lipp et al., 2001
5	Cm01 Cm02	5'-CACTACAAATGCCATCATTGCGATA-3' 5'-CTTATATAGAGGAAGGGTCTTGCGA-3'	220	Quist and Chapela, 2001
6	Mp3 Mp4	5'-TCATCCCTTACGTCAGTGGAGATAT-3' 5'-GATAAAGGAAAGGCCATCGTTGAAG-3'	155	Quist and Chapela, 2001
7	P35S-VoA P35S-VoB	5'-CCGACAGTGGTCCCAAAGATGGAC-3' 5'-ATATAGAGGAAGGGTCTTGCGAAGG-3'	168	Vollenhoffer et al., 1999; Ovesna et al., 2002
8	35S215F 35S319R	5'-ACT GAC GTA AGG GAT GAC GCA CAA-3' 5'-AGA CTG GTG ATT TCA GCG TGT CCT-3'	105	
9	35S215F 35S317R	5'-ACT GAC GTA AGG GAT GAC GCA CAA-3' 5'-ACT GGT GAT TTC AGC GTG TCC TCT-3'	103	
10	35S168F 35S319R	5'-ACG TTC CAA CCA CGT CTT CAA AGC-3' 5'-AGA CTG GTG ATT TCA GCG TGT CCT-3'	152	
11	35S168F 35S317R	5'-ACG TTC CAA CCA CGT CTT CAA AGC-3' 5'-ACT GGT GAT TTC AGC GTG TCC TCT-3'	150	
12	HMG-AF1 HMG-AR1	5'-GAA ATC CCT GAG CGA GTC GGT A-3' 5'-GCG ATG GCC TTG TTG TAC TCG A-3'	175	Hernandez et al., 2004

was re-suspended in 50 µl of distilled deionized water. The amount of DNA in each tube was determined by using a spectrophotometer (SmartSpec™ 3000, BIO-RAD, Richmond, CA).

Amplification of DNA

Primer sequences. Twelve primer pair sequences were used specifically for detecting the CaMV 35S promoter. Primer pairs 1 to 7 and 12 were taken from the literature, while primer pairs 8, 9, 10, and 11 were designed, using the sequence of the CaMV 35S promoter and the Oligo™ version 5.0 Primer Analysis Program. Information regarding the primers is listed in Table 1.

PCR analysis. The amplification of DNA was carried out in a final volume of 20 µl composed of 2 µl of 10x NH_4 buffer (1x), 0.6 µl of 50 mM $MgCl_2$ solution (1.5 mM), 0.4 µl of 10 mM dNTP mix (0.2 mM), 0.2 µl of DNA Polymerase (Biolase™, Bioline, Randolph, MA) (1 unit/reaction), 0.5 µl of forward and reverse primer (0.25 µM), 3 µl of DNA sample (10–50 ng/µL), and 12.8 µl of distilled deionized H_2O. Two control samples were used each time, a negative control containing water to verify that PCR reactions were free of contamination and a positive control containing plasmid DNA (pT102 construct) containing CaMV 35S promoter used in the transformation of maize embryos (Frame et al., 2002) was provided by Dr. Kan Wang (Agronomy, Plant Transformation Facility, ISU, Ames, IA). DNA amplification was performed on a PTC-100™ programmable Thermal Controller cycler (MJ Research, Inc., Watertown, MA). The quantity and quality of DNA were measured using a spectrophotometer. PCR amplification was carried out using 5.0 µl of DNA prep and the HMG-AF1/HMG-AR1 primer (high mobility group, corn specific primer). The steps are listed in Table 2.

Gel electrophoresis

Ten µl of PCR products with 1 µl of 10x DNA loading buffer (Eppendorf) were separated in a 3% plus ethidium Bromide precasted ReadyAgarose™ gels (BIO-RAD), in 1x TAE buffer during 90 min, using 75 v (power pac 300, and Mini-Sub® cell GT, BIO-RAD). Ten µl of HyperLadder V (Bioline, Randolph, MA) was used as the molecular size marker. The results were evaluated visually from the pictures of gels taken with a UV camera (BIO-RAD), analyzed in

TABLE 2. Conditions of thermocycler for DNA amplification of CaMV 35S promoter and corn genomic DNA.

Step	Primer pairs 1–7	Primers pairs 8–11	Primer pair 12
Denaturation	5 min/94 °C	3 min/94 °C	3 min/94 °C
Denaturation	30 s/94 °C	30 s/94 °C	30 s/94 °C
Annealing	60 s/50 °C	40 s/62 °C	30 s/62 °C
Extension	60 s/72 °C	40 s/72 °C	45 s/72 °C
Cycles (number)	30	40	40
Final extension	10 min/72 °C	5 min/72 °C	5 min/72 °C

Quantity One® Program (BIO-RAD), and printed with a video copy processor (Mitsubishi P91, Nagakakyo-City, Kyoto, Japan).

RESULTS AND DISCUSSION

DNA extraction from pollen and seed

Figures 1 and 2 are gel images of the PCR-amplified pollen DNA. The DNA extraction method used in this study was very successful at releasing the DNA from the pollen grains, as determined by the intensity of the bands. Figure 1 shows the genomic DNA from 18 pollen samples amplified using the primers HMG-AF1/HMG-AR1, a corn specific primer (Hernandez et al., 2004). The size of the amplicon was 175 base pairs. Most bands showed a high intensity, which also indicated that the primers have a high sensitivity for the detection of the pollen DNA. The high intensity of the bands is associated with the high specificity of the HMG primers for corn DNA. Rychlik and Rhoads (1989) reported that high specificity of the primers to the target DNA decreases mispriming and increases the number of copies of the amplicon. There was no amplification in lane 13, because it contained plasmid DNA which lacked corn-like sequences. These results confirm the specificity of primers to corn DNA. There are several methods available for DNA extraction and each method can yield significant differences in the amount and quality of DNA. The extraction method is a critical factor, since one method may work with one type of tissue or sample, but may be inappropriate for other types of samples. Generally, DNA extraction from pollen samples collected from greenhouse or growth chambers is easier, compared to the sample collected from fields. Field samples lose more moisture and can become contaminated by fungal DNA or dust. A wrong choice of extraction method can yield poor quality DNA; thus translating into higher rates of false positive and negative results and lower detection limits for PCR amplification (Pinero and Popping, 2003). The results from this study reveal that the DNA extraction method used was equally effective for pollen samples collected from the field and greenhouse (Figs. 1, 2, and 4). The extraction resulted in sufficient quantity of DNA for PCR amplification and PCR inhibitory substances were removed efficiently during the extraction process.

Detection of the transgene in pollen and seed DNA

The efficiency of the DNA extraction method and the efficiency of the primers to amplify DNA are related to the intensity of the bands in the respective gels. A set of primers reported in the literature and four new primers designed based on the 35 S sequences (Table 1), were screened for their efficiency to amplify DNA extracted from pollen of transgenic plants containing the 35S-CaMV promoter (Fig. 2). DNA bands showed more intensity in the experimental HilI transgenic corn variety (Fig. 2A) than the transgenic hybrid (Fig. 2B). We can speculate differences in the copy number of 35S sequences of both varieties. Based on the intensity of bands, primers P35S1/P35S2, P35SA/P35SB and P35S-aflu/P35S-ar1 were more efficient in amplifying transgenic pollen DNA, and primers P35S-cfr/P35S-cr4 were less efficient (Figs. 2A and 2B).

To test the efficiency of the different primers, seed DNA was extracted from eight transgenic hybrids and one non-transgenic hybrid. Figure 3 shows results from two of the seven primers. There were differences in the amplification of

FIGURE 1. DNA amplification of 18 non-transgenic corn pollen samples using the HMG-AF1/HMG-AR1 corn-specific primers. In the gel, Lane No. 1 represents the DNA Ladder, Lanes No. 2–12 and Lanes No. 14–20 contain the DNA from the corn pollen samples. Lane No. 13 contains plasmid DNA with the 35S-CaMV promoter.
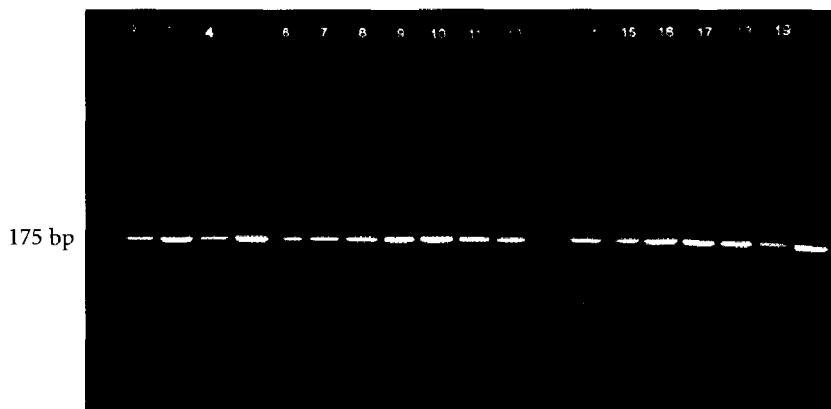

FIGURE 2. DNA amplification of two transgenic corn pollen samples. A: DNA from the experimental HiII transgenic corn variety; and B: DNA from DKC69-71 transgenic hybrid. Lane No.1: ladder; Lane No. 2: primers P35S1/P35S2; Lane No. 3: primers P35SA/P35SB; Lane No. 4: primers P35S-cfr/P35S-cr4; Lane No. 5: primers P35S-aflu/P35S-ar1; Lane No. 6: primers Cm01/Cm02; Lane No. 7: primers Mp3/Mp4; and Lane No. 8: primers P35S-VoA/P35S-VoB.
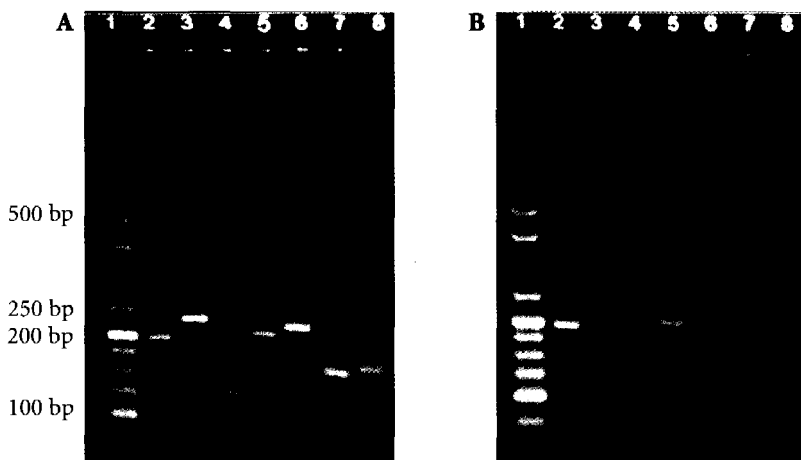
FIGURE 3. DNA amplification of eight transgenic corn hybrid seed samples. **A:** primers P35S1/P35S2; and **B:** primers P35S-aflu/P35S-ar1. Lane No. 1 contains the DNA ladder; Lane No. 2: hybrid FR1064×LH185Bt; Lane No. 3: hybrid LH245×LH185Bt; Lane No. 4: hybrid SGI928×HC50Bt; Lane No. 5: hybrid HC-SOBt×SGI905; Lane No. 6: hybrid TR7322×MBS1236Bt; Lane No. 7: DKC69-71hybrid; Lane No. 8: 4-NK7070Bt; Lane No. 9: Asgrow RX792 (non Bt-hybrid); Lane No. 10: ddH$_2$O; and Lane No. 11: plasmid DNA (pT102 construct).
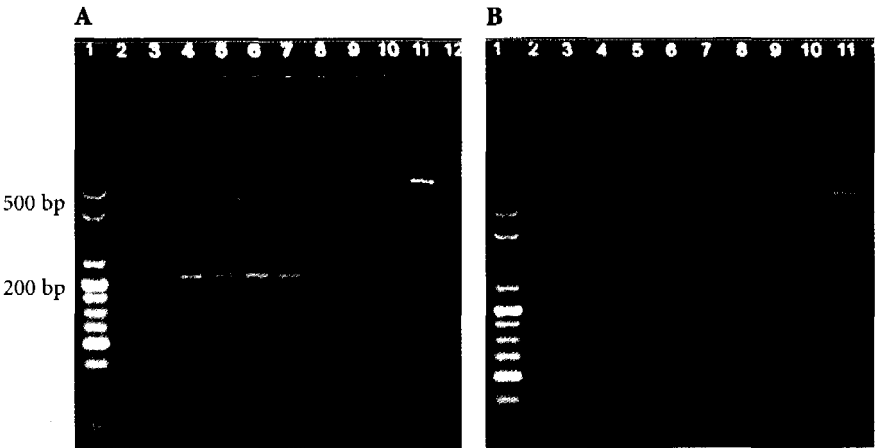

FIGURE 4. DNA amplification of seed and pollen of transgenic corn with primers 35S168F/35S317R. Lane No. 1: ladder; Lane No. 2: seed of hybrid FR1064× LH185Bt; Lane No. 3: double deionized (dd) H$_2$O; Lane No. 4: seed of hybrid LH245×LH185Bt; Lane No. 5: dd H$_2$O; Lane No. 6: seed of hybrid DKC69-71; Lane No. 7: dd H$_2$O; Lane No. 8: pollen of hybrid DKC69-71; Lane No. 9: dd H$_2$O; and Lane No. 10: plasmid DNA (pT102 construct).
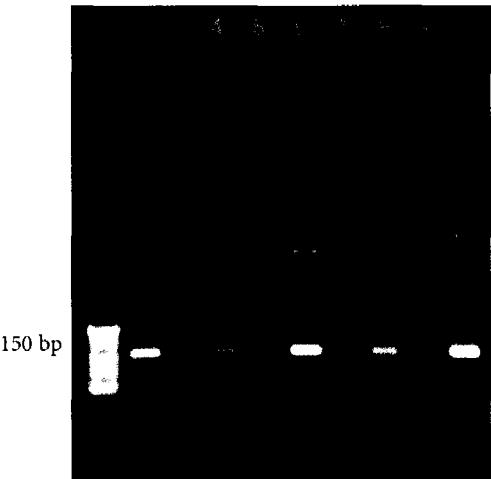
DNA in all hybrids, which may indicate differences in concentration of the transgene in the hybrids. The primer pair P35S1/P35S2 produced DNA fragment of higher intensity (Fig. 3A) than the fragment amplified by P35S-aflu/P35S-ar1 (Fig. 3B).

Four additional primers were designed in our lab to compare their efficiency with those reported in the literature. All primers were used in the amplification of DNA from transgenic pollen and seed. The most intense bands were generated by the 35S168F/35S317R primer combination in both transgenic pollen and seed DNA (Fig. 4), even though intensity was higher in the pollen samples. The size of the amplicon for 35S168F/35S317R (150 base pairs) was smaller than those of the primers reported in the literature (Table 1), with the exception of the amplicon of the primers P35S-cfr/P35S-cr4. The new primers 35S168F/35S317R showed higher efficiency based on band intensity to detect the 35S-CaMV promoter than primers reported in the literature.

There are several characteristics associated with the efficiency of the primers (Vanichanon et al., 2000). The length of primers should be at least 18–20 bases, an overall GC content of 40–60%, because higher amounts may result in mispriming (Dieffenbach et al., 1993; Innis and Gelfand, 1990), intraprimer-dimer and mainly interprimer-dimer formation by self-complementary reduces the annealing of the primers to the target sequence, decreasing yield and signal of the amplicon (Brownie et al., 1997; Rychlik and Rhoads, 1989; Vanichanon et al., 2000; Watson, 1989). Internal stability of the primer, given by a balanced representation of all four bases and by GC content toward the 5' or 3' end, should be low at the 3' end, because it may result in false priming due to a base pairing with non-target sequences, which results in a background smear of bands (Rychlik, 1995). In addition to the internal stability at the 3', a smear of bands may be caused by nonspecific amplification of target, due to differences in the melting temperatures (T_m) of the primers (Kim and Smithies, 1988; Dieffenbach et al., 1993). In this study, all primers have differences in the length in the overall GC content and in the trends of GC content toward the 5' or 3' end (Table 3).

Beasley et al. (1999) found that shorter primer length, higher primer GC content, and an increasing AT to GC trend toward the 3' end of the primers have strong effects on the sequence-tagged site failure. In the present study, the best designed primer that generated a 150 base pairs band (Fig. 4), is 24 bases in length, has 50% GC content, and a good balance in the trend of GC content toward the 5' and 3' end (Table 3).

CONCLUSIONS

The proposed pollen DNA extraction method was very effective in eliminating the PCR inhibitors from field samples and yielded sufficient quantity of DNA from pollen samples of transgenic and non-transgenic corn varieties. The amplification of pollen DNA using corn specific primers (HMG-AF1 and HMG-AR1) yielded an amplicon of 175 base pairs, indicating that DNA extracted from field samples was from the corn pollen. There were differences

TABLE 3. Name, length, and GC content of primers.

Primer Name	Length	GC content	GC content in 5'/3' half of the primer
P35S-1	19	47	5/4
P35S-2	20	50	5/5
P35S-A	20	50	5/5
P35S-B	20	50	4/6
P35S-cf3	21	52	6/5
P35S-cr4	25	36	5/4
P35S-aflu	21	43	43
P35S-ar1	20	55	7/4
Cm01	25	40	5/4
Cm02	25	44	4/7
Mp3	25	44	6/5
Mp4	25	40	4/6
P35S-VoA	24	58	8/6
P35S-VoB	25	40	4/6
35S215F	24	50	6/6
35S319R	24	50	5/7
35S215F	24	50	6/6
35S317R	24	50	5/7
35S168F	24	50	6/6 (GC at 3' end)
35S319R	24	50	5/7
35S168F	24	50	6/6 (GC at 3' end)
35S317R	24	50	5/7
HMG-AF1	22	55	5/7
HMG-AR1	22	55	7/5 (GC at 5' and 3' end)

in the efficiency of DNA amplification by the primers reported in the literature. Bands produced by the new primers 35S168F/35S317R were more intense than bands from primers published in the literature, especially in the pollen samples.

ACKNOWLEDGMENT

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