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Rapid Identification of Transformed Wheat Using a Half-Seed PCR Assay

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ABSTRACT

A simple, nondestructive PCR-based screening method has been developed for identifying putative transgenic soft white winter wheat (*Triticum aestivum* L.) carrying the coat protein gene of wheat streak mosaic virus. Removal of the endosperm end of individual seed provided sufficient material for DNA extraction and PCR. DNA from seed is more free of the secondary metabolites found in leaf tissue that can inhibit both PCR and restriction digests required for Southern analysis. The half-seed PCR assay has comparable accuracy to the leaf-tissue PCR assay and hence can be used as an accurate and rapid method for identifying transformed lines before planting. Germination of the remaining seed portion showed germination rates comparable to whole-seed controls. A slight delay in growth from the first-leaf through the first-tiller stage was observed in the half-seed-derived plants, as compared to plants grown from whole seed.

INTRODUCTION

Soft white winter wheat (*Triticum aestivum* L.) was transformed with the wheat streak mosaic virus coat protein (WSMV-CP) gene, and 69 putatively transformed lines carrying the WSMV-CP transgene were obtained. This resulted in approximately 1104 T₂ progeny plants to screen (20 plants/line). Assuming the T₂ populations each have one copy of the transgene, each dominant gene should segregate in a 3:1 phenotype ratio. Carrying these three phenotypic PCR-positive plants to the next generation would result in a minimum of 13248 plants, making further DNA screening unmanageable.

Challenge inoculations with virus and PCR assay for transgenes are common techniques used to identify transformed plants with virus resistance. Wheat plants in this study were me-

chanically inoculated with WSMV at the two-leaf stage, and symptoms are monitored for 20 days, after which resistant plants with reduced or no symptoms were identified and vernalized. When an adequate amount of tissue is available, DNA is extracted, usually 5–10 days after inoculation. Tissue is ground to a powder in liquid nitrogen, incubated at 65°C in extraction buffer for 20–30 min, purified using phenol:chloroform, and precipitated with 95% ethyl alcohol following Dellaporta's protocol (3). After precipitation, the DNA is vacuum-dried, rehydrated, and finally used for PCR. This process is time consuming and laborious.

Using PCR, transformed plant lines and transgenes can be readily identified (2,7,19). Although there are many techniques available for plant DNA extraction, most involve the use of leaf tissue, which may contain inhibitory secondary compounds, especially if the tissue is responding to infection (4–6, 10,11). Inhibitory compounds can be difficult to disassociate from genomic DNA and have been shown to inhibit PCR amplification (13,15). To reduce the total number of plants that must be screened for virus resistance in segregating populations, we developed an efficient method for isolating DNA from a half-seed for PCR assay. Here we show that DNA extracted from seed endosperm has few inhibitory compounds, resulting in improved DNA quality, with little or no PCR inhibition. This half-seed extraction technique provides sufficient DNA for PCR, while leaving a viable embryo for germination and future analysis.

MATERIALS AND METHODS

DNA Extraction

Seed samples from five parental lines of transgenic wheat were selected by PCR and Western analysis. Approximately 50% of each seed was excised from the endosperm end using a sterile scalpel. To prevent cross contamination, the scalpel was washed in ethanol and flamed between each sample. The endosperm end of the seed was placed within a piece of aluminum foil and pulverized using a hammer. Powder

generated from the half-seed was placed in a 1.7-mL centrifuge tube and 500 µL extraction buffer (7 M urea, 2% SDS, 5 mM EDTA) was added to the seed powder. After mixing the contents of each tube with a disposable pestle (Fisher Scientific, Pittsburgh, PA, USA), an equal volume of a 1:1 mixture of phenol:chloroform (chloroform/isoamyl alcohol at 24:1) was added, and the samples were vortex mixed and centrifuged at 8000× *g* for 5 min at room temperature. Approximately 400 µL of the supernatant from each tube were transferred to a new microcentrifuge tube where soluble carbohydrates were precipitated by slowly adding 0.3 volumes of 95% ethyl alcohol (16). After mixing the samples using inversion and then centrifugation at 8000× *g* for 10 min, the supernatant was removed and placed in a new microcentrifuge tube with 0.1 volume of 3 M sodium acetate and 0.7 volume of 2-propanol. This mixture was gently mixed by inversion and centrifuged at 10 000× *g* for 10 min at 4°C. The supernatant was discarded, and the pellet was washed with 1 mL 70% ethyl alcohol and centrifuged at 10 000× *g* for 10 min at 4°C. Pellets were dried using a SpeedVac® (Savant, Holbrook, NY, USA), and DNA resuspended in 50 µL TE buffer (0.01 M Tris-HCl, pH 8.0, 0.001 M EDTA).

Seed Germination and Seedling Development

The embryo half of the seed, along with control seeds were sown 2.4 cm deep in Sunshine Mix no. 1 (Sun-Gro Horticulture, Bellevue, WA, USA). An additional 200 control seeds were similarly halved and sown for a direct comparison and to eliminate any possible effect of the transgene. Greenhouse conditions were approximately 23°C/18°C day/night temperatures, and lighting was supplemented using metal halide and sodium vapor lamps providing a minimum of 200 W/m², with a 16/8 h light/dark photoperiod. When seedlings reached the five-leaf stage, approximately 100 mg tissue were collected from each plant and placed in a 1.5-mL microcentrifuge tube. The tube was immersed in liquid nitrogen, and the tissue was pulverized into a fine

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Table 1. Comparisons of Germination and Mean Growth Rates of Half versus Whole Seed Using Putative Transgenic Seed Carrying the WSMV-CP Gene and Control Seed Grown in the Greenhouse

Seed	No. Planted	% Germinated	Days to Two-Leaf ^a	Days to Five-Leaf ^a
T-Half ^b	40	95.0	24 ± 1.6	35 ± 2.1
T-Whole	40	97.5	20 ± 2.0	30 ± 1.5
Half	200	97.0	25 ± 3.2	35 ± 2.2
Whole	200	97.5	20 ± 3.2	30 ± 2.1

^aMean ± SD
^bT, transgenic seed

powder using a disposable pestle. DNA was extracted in the same manner as described for half-seeds. Extracted DNA was quantified using either fluorimetry (Bio-Rad Laboratories, Hercules, CA, USA) or densitometry (Alpha Innotech, Fort Jefferson, NY, USA) using mass ladder standards (Invitrogen, Carlsbad, CA, USA).

PCR

PCR was performed using 20-mer primers (Invitrogen) specific to the WSMV-CP gene, which amplified a 499-bp fragment of the transgene. The forward primer sequence was 5'-C-TCATTGTCCTTGCTGCAT-3', and the corresponding reverse primer was 5'-ATCAGGTTCTGGAGCAGCAC-3'. A 50-μL PCR mixture containing the manufacturer's supplied buffer (2.0 mM MgCl₂, 100 pM each dNTP, 200 nM each primer, and 1 U Taq DNA polymerase) was used per sample. Sample DNA (0.2 μg) was added to the microcentrifuge tubes and overlaid with 25 μL Chill-out™ (MJ Research, Waltham, MA, USA). A three-step PCR was performed using a PHC-3 thermal cycler (Techne, Princeton, NJ, USA): initial denaturing at 94°C for 3 min, followed by 35 cycles of 63°C for 20 s, 72°C for 30 s, and 94°C for 20 s. Final elongation was carried out at 72°C for 10 min, after which 1.0 μL each PCR product was analyzed by 1% agarose gel electrophoresis.

RESULTS AND DISCUSSION

Samples from both seed endosperm and leaf tissue yielded from 0.36 to

1.10 μg/μL, with total yields ranging from 18 to 55 μg. The DNA extracted from seed appeared to provide improved PCR screening of transgenic plants when compared to DNA extracted from leaf tissue on the basis of less smearing (Figure 1). More PCR-positive samples were identified by half-seed PCR than by leaf PCR (Figure 2). When seedlings from the embryo halves of the assayed seeds reached the Feeke's two-leaf stage, they were inoculated with WSMV, and disease symptom development was followed for 20 days. After this period, all of the PCR-positive plants showed no symptoms of wheat streak mosaic.

Germination rates of the half seed were not significantly different from the whole seed of the transgenic lines evaluated. Germination incidences among transgenic embryos were 95% and 97.5% for the half and whole seed, respectively. Control half and whole seed sown along with the transgenic lines had comparable germination frequencies of 97% and 97.5% for the half and whole seed, respectively (Table 1). Such differences were negligible because germination frequencies observed in our greenhouse are typically between 94% and 100% for both transgenic and control seed. Comparable germination frequencies using half seeds have also been reported previously in rice (1).

In our study, plants from half seeds exhibited narrower leaves compared to plants from whole seeds. In addition, development from emergence to the five-leaf stage was delayed by an average of five days. The height was the same in both the half- and whole-seed plants at this stage, however. Beyond the production of the second tiller, both

Figure 1. PCR of DNA extracted from either leaf or seed. Lanes 1 and 10, 100-bp ladder; lane 2, non-transformed Daws DNA; lane 3, Daws genomic DNA with 10 pg plasmid containing the WSMV-CP. Lanes 4–6 were extracted using leaf tissue, and lanes 7–9 were extracted from half seeds. Note smearing in lanes where leaf DNA was used for PCR.

Figure 2. Reactions from six lines containing the WSMV-CP transgene. Lanes M, 100-base ladder; lane 1, positive control; lanes 2–7, reactions from half-seed genomic DNA; lane 8, a no template control; and lanes 9–14, reactions from leaf genomic DNA.

groups of plants appeared to be phenotypically equivalent. No exceptional differences in seed yield or quality were observed between plants derived from half or full seeds.

Although DNA extraction from seeds share components with several “quick-leaf” extractions for PCR analysis, extra steps are usually necessary to ensure acceptable results when extracting DNA from seed. Simple extraction protocols that require single tubes are often sufficient for extraction from the relatively succulent leaf tissue of plants such as tomato, tobacco, and *Arabidopsis* (9). In contrast, seeds are often comprised of compact, desiccated tissue containing large amounts of storage molecules. Most seeds need treatments such as the

use of liquid nitrogen, heating, and mechanical grinding for the proper release of DNA from these tissues.

DNA derived from the seed of red clover, cotton, French bean, soybean, maize, rice, and wheat have been used for RAPD (1,12,14) and RFLP analyses (1,8) and for the identification of gene families for marker-assisted breeding (1,17,18). Wang et al. (18,19) used whole-imbibed cottonseed to extract DNA and Tamhannkar et al. (17) extracted DNA for RFLP analysis from whole French bean seeds after eight days of imbibement. Both extraction processes used a standard CTAB protocol for plant tissue. In the half-seed assay using the endosperm end from rice, Chunwongse et al. (1) incubated the

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half-seed in PCR buffer with proteinase K for 1 h, boiled for 5 min, and then centrifuged. The supernatant was then used for PCR amplification, and the embryo end of the seed was sown and grown successfully, as in our study. While the method of Chunwongse et al. (1) maintained a viable embryo, their technique was lengthy and provided only a crude DNA extract for PCR. Our half-seed assay has fewer steps, takes less time, and the DNA is precipitated and rehydrated to a quantifiable concentration. DNA can then be used for PCR with consistency. Potentially, Southern blot hybridization can also be carried out with the purified DNA.

This is the report of rapid DNA isolation from half wheat seed and to use the viable embryo for obtaining plants containing transgenes. Because seed screening can take place before sowing, this technique should aid in screening putative transgenic wheat lines and save time, expense, and greenhouse space.

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