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Protocols

Serial Extraction of Endosperm Drillings (SEED) - A Method for Detecting Transgenes and Proteins in Single Viable Maize Kernels

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Abstract. We have developed a method for detecting a transgene and its protein product in maize endosperm that allows the kernel to be germinated after analysis. This technique could be highly useful for several monocots and dicots. Our method involves first sampling the endosperm with a hand-held rotary grinder so that the embryo is preserved and capable of germination. This tissue is then serially extracted, first with SDS-PAGE sample buffer to extract proteins, then with an aqueous buffer to extract DNA. The product of the transgene can be detected in the first extract by SDS-PAGE with visualization by total protein staining or immuno-blot detection. The second extract can be purified and used as template DNA in PCR reactions to detect the transgene. This method is particularly useful for screening transgenic kernels in breeding experiments and testing for gene silencing in kernels.

Key words: ELISA, gene expression, PCR, transgene, *Zea mays* L.

Introduction

Because of recent advances in plant transformation research, transgenic crops are being incorporated into many breeding and research programs. A major difficulty with working with transgenic plants is gene silencing (Kumpatla et al., 1998). While transgenes are often inherited in a predicable manner, the expression of the transgene often varies in different lines due to position effects or epigenetic effects. Thus, it is necessary to monitor inheritance and expression of the transgene each generation to verify that the transgene functions properly in the conditions tested. Inexpensive, high-throughput methods are needed to meet the demand of breeding programs for screening large numbers of plants.

We have developed a method that allows detection of transgenes and their protein products in the endosperm of individual maize kernels. The embryo is not

| Grind to sample endosperm | ➡ | Plant seed |

Endosperm drillings ⬇   Sampled kernel

| Extract with protein extraction buffer |

⬇

| Centrifuge | Supernatant ➡ | SDS-PAGE protein stain or immuno-detect |

Pellet ⬇

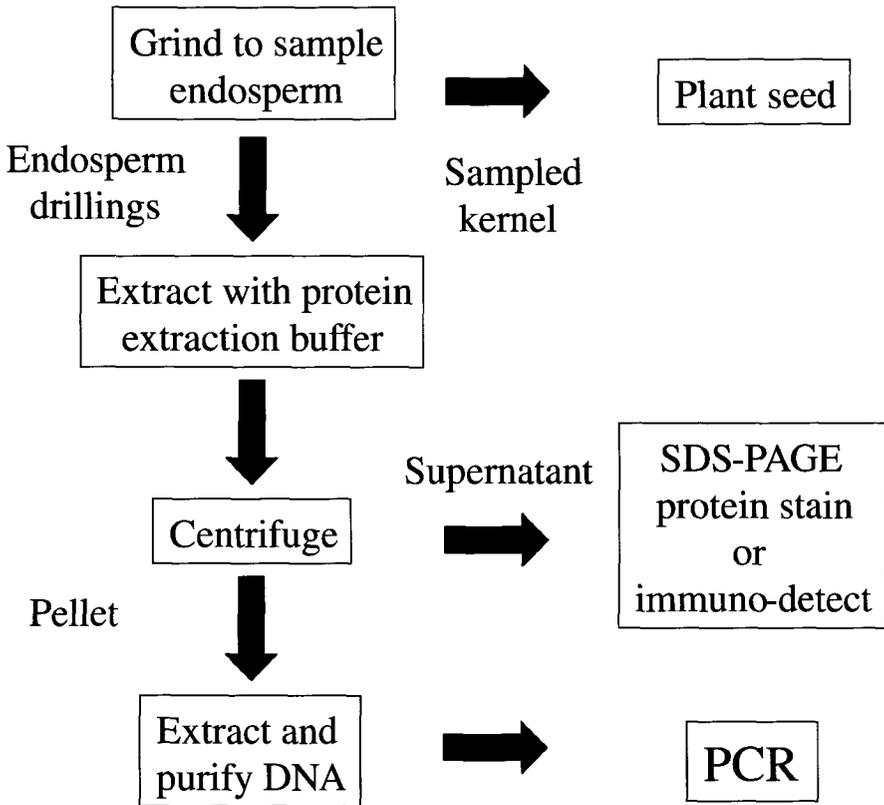| Extract and purify DNA | ➡ | PCR |

Figure 1. Schematic diagram of the SEED method.

damaged so that the kernels can be analyzed, stored, and subsequently germi-
nated. Nonlethal sampling methods that retain seed viability are frequently used
to screen kernel traits. Our method couples nonlethal sampling with serial extrac-
tion of the excised tissue to obtain extracts suitable for several types of analyses.
Serial extraction was one of the first methods used to separate and classify seed
components (Osborne, 1924); it is based on the differential solubility of the com-
ponents being separated. Coupled with microsampling and analysis methods, se-
rial extraction is a useful tool in molecular breeding.

Materials and Methods

The protocol is outlined schematically in Figure 1. All liquid handling steps are
carried out in 96-well format, which lends itself to automation for
high-throughput. All chemicals are molecular biology grade or equivalent. The
protocol presented here is optimized for analysis of the wheat Glu1-Dx5
transgene in maize. The parameters of SDS-PAGE, ELISA, PCR, and
immuno-blotting will require optimization for detection of other transgenes and
proteins.

Grinding apparatus

Maize kernels are ground using a hand-held rotary grinder (Sears Craftsman variable speed rotary tool, model #61053, Sears Roebuck and Co., Chicago, Illinois, USA) with a flexible shaft attachment. A 3 mm^2 patch of pericarp is removed with sand paper. The kernels are then placed on a small piece of weighing paper and kept in place by the operator's thumbnail. The kernels are held embryo side down with the pedicel underneath the thumb. The rotary grinder is used at approximately 50% full speed with a #105 Dremel grinding bit. The endosperm is carefully ground to avoid damaging the embryo or removing too much of the endosperm. Typical yields range from 10-20 mg of finely ground endosperm. This powder is transferred to a 1.5 mL round-bottom 96-well plate using a small glass funnel. Drill bits, funnels and fingers are thoroughly washed between samples to avoid contamination. A fresh sheet of weigh paper is used for each kernel. To increase the efficiency of the process, several funnels and drill bits are used so that they do not have to be washed between every kernel.

Protein extraction for SDS-PAGE and immuno-blot analysis

Protein extraction buffer (0.0625N Tris-HCl, [pH 6.8], 3.3% (W/V) SDS, 5% (v/v) 2-mercaptoethanol, 10% glycerol, 0.002% Bromphenol blue) is added to preweighed samples in a ratio of 10 to 1 μL/mg. One hundred to two hundred microliters of protein extraction buffer is added to 10-20 mg of ground endosperm in the ratio of 10 μL buffer per 1 mg ground endosperm. The 96-well plate is mixed for 10 min using a vortex mixer followed by 2 h of shaking at 1400 RPM at room temperature. The 96-well plate is then centrifuged for 5 min at 4000 g-force; the supernatants are transferred to a new 96-well plate. These extracts are heated to 95°C for 5 min and stored at -20°C before analysis. The extracted endosperm pellet is used for DNA extraction.

SDS-PAGE

Extracted proteins are separated by electrophoresis on a 12% SDS-PAGE (Acrylamide/bis 37.5:1) gel (Laemmli, 1970) using a Mini-Protean II Electrophoresis Cell from BIO-RAD with a 20-well comb. Five microliters of protein extract is loaded in each well. Electrophoresis is carried out at 100 volts for 1.5 h. Gels are stained with 0.1% Coomassie Blue-R-250 in 1% acetic acid and 40% ethanol for 0.5 h and destained with 40% ethanol and 10% acetic acid for 1-3 h.

Immuno-blot analysis

Immuno-blot detection is carried out using a Bio-Rad Trans-Blot apparatus according to the manufacturer's directions. Proteins are detected using a monoclonal antibody that specifically binds to the transgene product.

DNA extraction

DNA is extracted and purified from the endosperm pellet remaining in the 96-well plate after extraction of the proteins for SDS-PAGE. The DNA is extracted in 300 μL of cell lysis buffer from the Puregene DNA purification kit (Gentra), and

purification is carried out using the Puregene protocol according to the manufacturer's directions. Since the purification is carried out in microplate format, centrifugation is conducted at 4000 *g*-force for 5 min in a swinging bucket microplate rotor. DNA resulting from this procedure is resuspended in 100 μL of 1 mM Tris HCl, (pH 8.0), 0.1 mM EDTA. The DNA is allowed to resuspend by first heating to 65°C for 60 min, then shaking at 300 RPM and 50°C overnight in a shaking incubator.

Polymerase chain reaction

The transgene is detected by PCR using 5 μL of the purified DNA as template and primers specific to the transgene. A touchdown PCR protocol is used (Senior and Huen, 1993). Products were separated on 2% Metaphor (FMC Bioproducts) agarose gels and visualized by UV fluorescence of the ethidium bromide-stained DNA.

Alternative protocol for ELISA analysis

An alternative protocol can be used to detect the protein by ELISA rather than immuno-blot analysis. The protein extraction procedure for ELISA analysis is the same as that used for SDS-PAGE and immuno-blot analysis, except that our transgene product requires a different protein extraction buffer (70% ethanol, 61 mM Sodium Acetate, and add 5% [v/v] 2-mercaptoethanol before use) and 20-30 mg of endosperm is extracted in a ratio of 10 μL to 1 mg. The resulting extract can be analyzed by SDS-PAGE and immuno-blot detection as well. The extracted endosperm pellet was dried overnight at 4°C and then used for DNA extraction.

ELISA analysis is conducted according to standard protocols (Harlow and Lane, 1999). Standards of purified transgene product were included in a range of 5-120 ng/50 uL.

Seed germination

Because the sampling process damages the pericarp, we took precautions to protect the seeds from fungal infection. Before planting in sterile soil, the seeds were treated with Chlorothalonil (tetrachloroisophthalonitrile 0.087%, Fung-Onil, Earl May). Seeds were germinated in 3" peat pots in the greenhouse and transplanted to the field.

Results

We have applied this method to analyze over 2000 kernels from populations of maize segregating for a transgene from wheat, the HMW-Glutenin 1Dx5 gene (Anderson et al., 1989). In wheat, expression of the glutenins is normally tissue specific (Shewry, 1995) with expression confined to the endosperm. We anticipated a similar expression pattern in maize given the genetic similarity of these organisms (Bennetzen and Freeling, 1993).
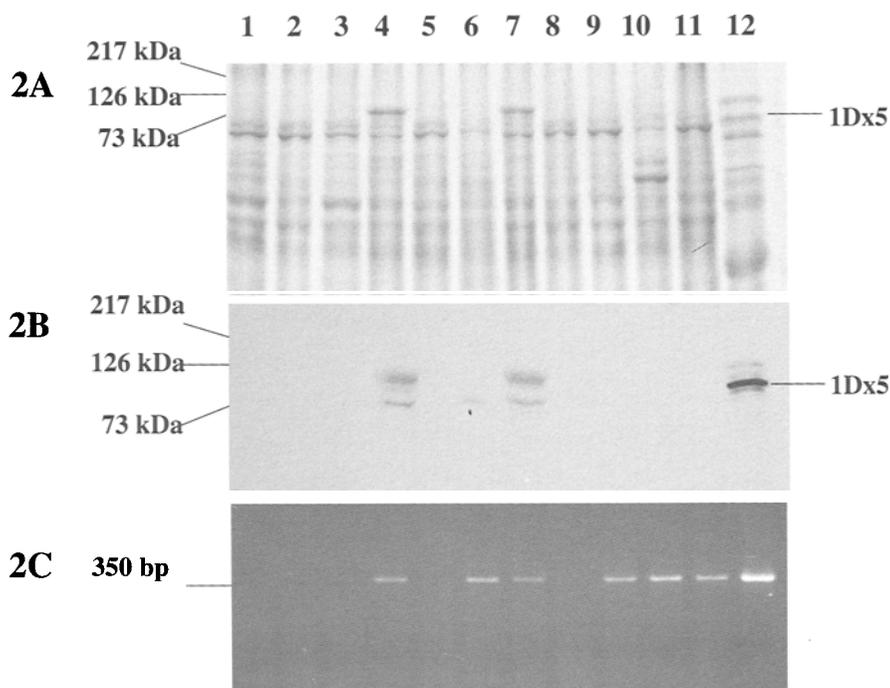
Figure 2. (A) Characterization of proteins from endosperm drillings by SDS-PAGE with Coomassie Brilliant Blue staining. Lane 1: nontransgenic maize kernel. Lanes 2-11: transgenic maize kernels. Lane 12: wheat variety L88-6, which contains the 1Dx5 subunit. The same endosperm drillings were used to make Figures 2B and 2C. (B) Immuno-blot detection of SDS-PAGE-separated proteins. Lanes are labeled as in Figure 2A. (C) Detection of the transgene by PCR, agarose gel electrophoresis, and ethidium bromide staining. Lanes are labeled as in Figures 2A and 2B, except Lane 12 contains plasmid DNA of the construct used in transformation. DNA was purified from the same endosperm drillings used to make Figures 2A and 2B.

Protein analysis

In order to determine if the transgene was functional in maize endosperm, we tested for the accumulation of the protein it encoded using SDS-PAGE (Laemmli, 1970). This method is sensitive enough that the drillings from a single mature kernel (about 20 mg) can be analyzed. The transgene product was sufficiently abundant to be visible by Coomassie Brilliant Blue staining (Figure 2A). In order to confirm that the bands in the Coomassie-stained gel were the product of the transgene, we ran duplicate gels and visualized one gel using immuno-blot detection (Figure 2B) using a monoclonal antibody specific to wheat high molecular weight glutenin subunits. A band in the position of the prominent Coomassie-stained band was detected by this method, as well as a weaker band just below it. This lower band may be a product of proteolysis.

The variation of the protocol utilizing ELISA analysis to detect the transgene product is valuable because it yields a quantitative measure of the target protein (Table 1). Using ELISA, we were able to detect the transgene product in kernels that were scored negative by SDS-PAGE (e.g. Table 1, Sample 5).

Table 1. Analysis of segregating F_4 kernels using ELISA in the SEED protocol[a].

Samples	1Dx5 concentration (ng/mg seed ± SD)[b]	SDS-PAGE	PCR
1	-14.5 ± 3.6	-	-
2	50.3 ± 7.3	+	+
3	-9.3 ± 3.6	-	+
4	55.5 ± 7.3	+	+
5	6.2 ± 3.6	-	+
6	42.5 ± 3.6	+	+
7	55.5 ± 7.3	+	+
8	-9.3 ± 3.6	-	-
9	-11.95 ± 0.0	-	-
10	-9.3 ± 3.6	-	-
11	-9.3 ± 3.6	-	-
12	-9.3 ± 3.6	-	-
13	55.5 ± 7.3	+	+
14	-6.7 ± 0.0	-	-
15 nontransgenic corn	-9.3 ± 3.6	-	-
16 wheat	50.3 ± 7.3	+	+
17 plasmid			+

[a]These samples are different from those used in Figure 2.
[b]We interpret negative values to indicate no detectable expression.

Genotype analysis

A common goal is to develop transgenic lines that are homozygous for the transgene. As we advance plants in this breeding program, there are at least two reasons why a kernel may not express the transgene. First, it may not have inherited the gene and, second, the gene may have been silenced. To distinguish between these two possibilities, it was necessary to determine if the transgene was present in the kernels lacking expression of the transgene as measured by SDS-PAGE. After extraction for protein analysis, the kernel drillings contained enough DNA to extract and use for PCR analysis. We found that all kernels expressing the protein were positive in the PCR analysis, but some kernels that did not contain the protein contained the gene, while others did not. We concluded that kernels lacking both the gene and the protein did not inherit the transgene; while, in kernels containing the gene without detectable protein, the transgene was silenced. Caution is needed when interpreting PCR results, because the endosperm tissue will normally be contaminated with small amounts of pericarp tissue, which could contain the transgene when the corresponding endosperm does not. Because pericarp tissue is present in very small quantities relative to endosperm, it should be possible to adjust the PCR conditions to distinguish between bands resulting from endosperm DNA and those resulting from pericarp DNA by comparing band intensities.

Germination of analyzed seeds

The sampled seeds were planted using sterile soil in a greenhouse before transplanting to the field. Of 236 seeds planted, 191 (81%) germinated. Our normal

germination rate is about 90%, so we conclude that our sampling procedure has a minor impact, if any, on the ability of sampled seeds to germinate in carefully controlled conditions.

Discussion

In most high-throughput seed analysis strategies, grinding the seed requires a substantial amount of labor. The serial extraction strategy employed by this method minimizes the grinding requirement by using the same ground material for several types of analysis. This method is particularly useful for the analysis of transgenes targeting cereal kernel quality traits, which often direct expression specifically in the endosperm. The method is applicable to any transgene containing a unique DNA sequence and encoding a product that is detectable in seeds by ELISA, immuno-blotting or total protein staining of SDS-PAGE. ELISA has the advantages of being quantitative and the throughput is higher than with immuno-blot analysis. Nontarget proteins that react with the antibody can interfere in this type of assay, so it is important to verify the specificity of the antibody. The throughput of the method is sufficiently high to be useful in a breeding program. One person can analyze 192 kernels in 4-5 d (two 96-well plates). Grinding the tissue takes the majority of the time.

Nondestructive seed sampling methods have been in use for many years with several plant species because they are rapid and planting decisions can be based on the results of the analysis. This method includes these advantages and, in addition, the serial extraction allows it to give information both about the inheritance and the expression of the transgene. This is particularly useful in situations where gene silencing is common.

We have tested the method with corn; however, it also should be applicable to other cereals. It will be interesting to determine if this method could be applied to large-seeded dicots by sampling the cotyledons in place of the endosperm.

Acknowledgments

References

Anderson OD, Greene FC, Yip RE, Halford NG, Shewry PR and Malpica-Romero JM (1989) Nucleotide sequences of the two high-molecular-weight glutenin genes from the D-genome of a hexaploid bread wheat, *Triticum aestivum L.* cv Cheyenne. Nucleic Acids Res 17: 461-462.

Bennetzen JL and Freeling M (1993) Grasses as a single genetic system: Genome composition, colinearity and compatibility. Trends Genet 9: 259-261.

Harlow E and Lane D (1999) Using Antibodies: A Laboratory Manual, pp 495, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Kumpatla SP, Chandrasekharan MB, Iyer LM, Li G and Hall TC (1998) Genome intruder scanning and modulation systems and transgene silencing. Trends Plant Sci 3: 97-104.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Osborne TB (1924) The Vegetable Proteins, pp 154, Longmans Green and Co., New York.

Senior ML and Heun M (1993) Mapping maize microsatellites and polymerase chain reaction confirmation of the targeted repeats using a CT primer. Genome 36: 884-889.

Shewry PR (1995) Plant storage proteins. Biol Rev Camb Philos Soc 70: 375-426.