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S. R. Kata · B. H. Taylor · A. J. Bockholt · J. D. Smith

Identification of *opaque-2* genotypes in segregating populations of Quality Protein Maize by analysis of restriction fragment length polymorphisms

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Abstract Quality Protein Maize (QPM) is a name given to genetically modified *opaque-2* maize with hard endosperm. The *opaque-2* mutation conditions a reduction in the amount of zein seed storage protein; zeins are deficient in the essential amino acids lysine and tryptophan, and mutant seed have a higher nutritional value. To utilize the potential of *opaque-2* maize, elite inbreds can be converted to *o2/o2* forms and subsequently to hard endosperm *opaque-2*. Since *opaque-2* is recessive and endosperm specific, conventional backcross procedures to convert elite inbreds to *opaque-2* forms are inefficient. To alleviate this problem, a marker-assisted selection procedure was developed for the Texas A&M University Quality Protein Maize breeding program. Hybridization of an *O2* cDNA probe to blots of DNA from plants carrying *O2* and *o2* alleles showed that restriction fragment length polymorphisms (RFLPs) exist between the W64A *o2* allele and *O2* alleles of Mo17 and TX5855 inbred lines. To identify the *opaque-2* genotypes in segregating populations, an RFLP marker assay combining the *O2* cDNA probe and *Hin*dIII-digestion of genomic DNA was developed. The effectiveness of the *O2* RFLP marker assay was tested under field conditions using F_2 and backcross populations of several hard endosperm *opaque-2* lines. A comparison of the genotypes identified by RFLP analysis with the seed phenotypes of the next generation indicated that this procedure is accurate and can be used for identifying *O2/O2*, *O2/o2*, and *o2/o2* genotypes of individual juvenile plants in breeding populations.

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S. R. Kata · A. J. Bockholt · J. D. Smith (✉)
Department of Soil and Crop Sciences,
Texas A&M University,
College Station,
Texas 77843, USA

B. H. Taylor
Department of Biology
Texas A&M University,
College Station,
Texas 77843, USA

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Introduction

Zeins (prolamines) constitute about 60–70% of the maize endosperm protein and are low in the essential amino acids lysine, tryptophan, and methionine (Mertz et al. 1964; for review see Tsai 1983). The *O2* gene codes for a protein that is required for the transcription of the 22-kDa zein structural genes (Soave et al. 1986; Schmidt et al. 1990), and 22 kDa zein synthesis is almost completely repressed in *o2/o2* maize endosperm (Kodrzycki et al. 1989). Since the 22 kDa zeins account for 25% of the total endosperm protein, *opaque-2* maize has higher percentages of lysine and tryptophan and is more nutritionally balanced than normal maize.

Despite the nutritional advantages, *opaque-2* maize varieties have not been widely accepted due to agronomic limitations, e.g., chalky endosperm, slow drying at harvest, reduced yield, increased disease susceptibility, and excessive mechanical damage during harvesting and handling (for review, see Glover and Mertz 1987). To circumvent these problems, breeding strategies were devised to derive high quality maize carrying the *opaque-2* mutation. Researchers at CIMMYT were successful in developing hard endosperm *opaque-2* maize varieties by recurrent selection for hardness, high lysine, and high tryptophan. These new varieties were called Quality Protein Maize (QPM) (Villegas et al. 1992). A research group at Natal, South Africa was also successful in releasing High Lysine Maize (HLM) (Gevers and Lake 1992). In these varieties, most of the problems associated with *opaque-2* were reduced or eliminated. Modified hard endosperm *o2* maize is similar to conventional maize, but the kernels are homozygous for the *o2* allele. Some QPM varieties have higher percentages of lysine and tryptophan in the whole grain than non-mutant maize and have a protein biological value (BV) 90% that of milk (Glover 1988). The varieties that were released by CIMMYT (for review, see Glover and Mertz 1987) were

subsequently used as source material for QPM traits in several other breeding programs.

There are, however, limitations on the progress of QPM breeding that can be expected from conventional breeding programs. The *O2* gene is expressed only in endosperm tissue, and the *o2* plant development is normal. The most rapid backcross procedure (commonly used for the transfer of specific genes) for converting a standard inbred to an *opaque-2* form requires at least 7 generations: initial cross, 5 backcrosses without testcross identification of *opaque-2,* and a selfing generation to establish the *opaque-2* lines. A large population size is required, since less than 1% of the selections made during conversion will be *opaque-2* in the seventh generation. More conventional backcross procedures that include testcrosses in alternate generations require at least 12 generations for conversion. The initial selection is based on the *opaque-2* or QPM phenotype, and large numbers of selections are required for each selection cycle because agronomic characteristics must also be evaluated. Because the kernel phenotypes cannot be identified in immature plants, selections for plant and agronomic characteristics and crosses for early testing of combining ability must be made prior to seed character identification. The phenotypic appearance of *opaque-2* kernels segregating in normal ears may be relatively easy to identify, but this procedure is frequently unreliable because the relative opacity of the *opaque-2* kernels varies with different genetic backgrounds. Thus, conventional breeding programs, even though successful, are highly inefficient for converting standard inbreds to *opaque-2* forms.

The advantages of molecular markers over morphological markers for genetic and breeding applications have been previously described (Tanksley et al. 1989; Walton and Helentjaris 1987; Stuber 1992). If molecular markers like restriction fragment length polymorphisms (RFLPs: Botstein et al. 1980) are used to identify the *opaque-2* genotypes, the efficiency of the backcross method of breeding can be enhanced. The use of molecular markers to identify heterozygous plants prior to selection would greatly enhance the efficiency of backcrossing without testcrossing. Population size could be reduced to a few plants per generation, and the generations needed for inbred conversion could be reduced by half. Because the target genotype is identified in advance, seed and plant characteristics may be selected simultaneously. The objectives of the present study were to develop an *O2* RFLP marker assay (a specific DNA probe/restriction enzyme combination is referred to as an RFLP marker), and to test its effectiveness in the identification of *O2/O2, O2/o2*, and *o2/o2* genotypes of individual juvenile plants in segregating populations.

Materials and methods

Plant materials

Two hard endosperm *o2/o2* populations, POP20 and POP22 from Hans Gevers, Natal, South Africa, and one CIMMYT QPM population, POB67, were used as *opaque-2* source material. The *o2* mutant allele in all these populations is believed to be the original reference mutant that was found in inbred W64A (Mertz et al. 1964). The inbred line TX5855 was used as the wild type source (O2/O2). F_1 plants were generated at Homestead, Florida (November, 1991) by making the following crosses: TX5855 × POP20, TX5855 × POP22, TX5855 × POB67. F_2 and backcross populations (BC) were obtained (June, 1992) by selfing and backcrossing to both the parents (Table 1). The F_2 and BC populations were planted on the Texas A&M University Research Farm, College Station, Texas (March, 1993). Individual juvenile plants were tagged, and leaf samples from young tissue (3–4 weeks after sprouting) were collected in ziploc bags, stored on ice, and transported to the laboratory. Individual leaf samples were cleaned, frozen in 50-ml Falcon tubes with liquid N_2, and stored at –80 °C until used.To compare the predicted genotype with the progeny seed phenotype, each plant was self pollinated and the individual ears were tagged and labelled. The seed phenotypes of the individual ears were identified using a light box. To illustrate the different phenotypes, kernel photographs were taken with an Olympus SZH-10 stereoscope with dark field illumination.

DNA Isolation and quantitation

Total DNA was isolated from individual leaf samples using the cetyl trimethyl ammonium bromide (CTAB) extraction procedure first described by Murray and Thompson (1980) and modified by Taylor et al. (1993). Approximately 1 g of frozen tissue was used for each DNA extraction. The concentration of DNA was determined using a TKO 100 DNA Mini-Fluorometer according to the protocol provided by the manufacturer (Hoefer Scientific Instruments, San Francisco, Calif.).

Plant genomic DNA digestion, electrophoresis, and blotting

The DNA digestion with restriction endonucleases was performed with buffers provided by the supplier (Boehringer Mannheim). To improve the digestion, 4 m*M* spermidine was added to the DNA, and 3 g of genomic DNA was then digested with 10 units of enzyme for at least 90 min at 37 °C. The digested DNA was electrophoresed on 0.8% agarose gels in TBE buffer (Sambrook et al. 1989). A $[^{32}P]$-labelled 1-kb DNA ladder (GIBCO/BRL, Bethesda, Md) was used in all the gels as a fragment size marker. Known genotypes were used as standards [TX5855 (*O2/O2*) and W64A*o2* (*o2/o2*)]. The gels were stained in water containing 250 ng/ml of ethidium bromide for 15 min, destained in water for 15 min and photographed under UV illumination to confirm uniform loading and digestion.

The gels were treated with 0.25 *N* hydrochloric acid for 5–10 min to depurinate the fractionated DNA (for efficient transfer of larger DNA fragments), denatured in 0.4 *N* sodium hydroxide (NaOH), and then blotted to polysulfone membrane (Biotrace, Gelman Sciences) using an alkaline capillary transfer with 0.4 *N* NaOH (Reed and Mann 1985; Sambrook et al. 1989) for 18–20 h. The membranes were oven dried at 65 °C (without vacuum) for 45 min.

Table 1 Generation of F_2 and BC populations

F_2 population	BC population[a]	BC population[b]
(TX5855/POP20)×(TX5855/POP20)	(TX5855/POP20)×(POP20)	(TX5855/POP20)×(TX5855)
(TX5855/POP22)×(TX5855/POP22)	–	(TX5855/POP22)×(TX5855)
(TX5855/POB67)×(TX5855/POB67)	(TX5855/POB67)×(POB67)	(TX5855/POB67)×(TX5855)

[a] F_1 backcrossed to *o2/o2*
[b] F_1 backcrossed to *O2/O2*

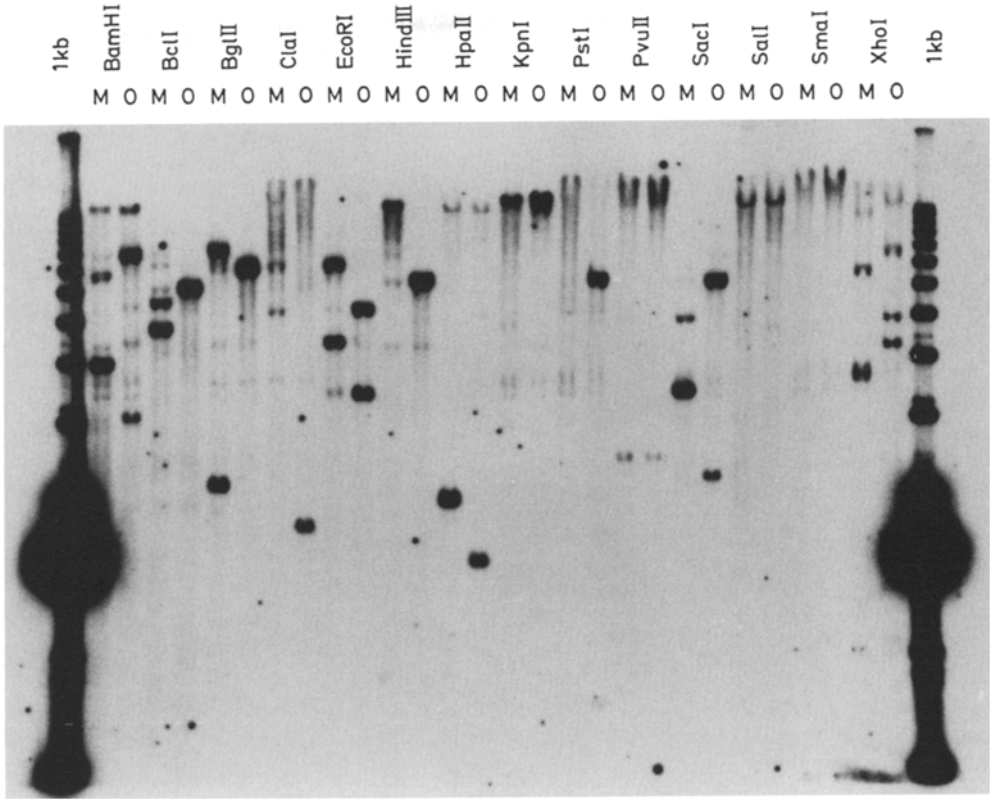

Fig. 1 RFLP analysis of the Opaque-2 locus. Genomic DNA (5 µg) from seedlings of Mo17 (*M*) and Mo17 *o2/o2* (*O*) was digested with the restriction endonucleases indicated above each pair of lanes. DNA was fractionated on 0.8% agarose and blotted to Magnagraph nylon membrane (MSI). The blot was probed with a full-length *Opa-que-2* cDNA clone (including vector) radiolabelled by random primer labelling. *1 kb* 1-kb size marker (BRL). Distinct bands in the marker lane range from 3 kb to 12 kb

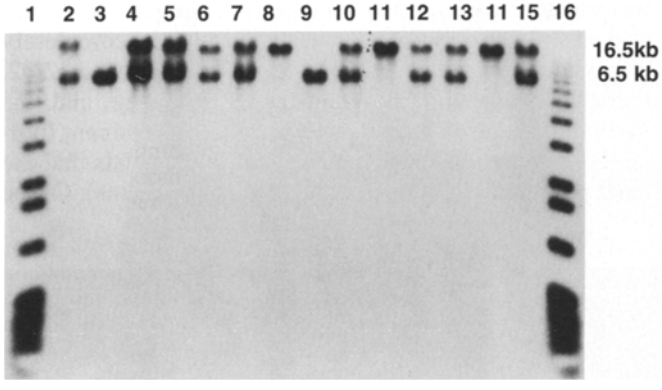

Fig. 2 RFLP analysis of individual juvenile plants of a BC population. Genomic DNA (3 µg) from individual seedlings of each BC population was digested with the restriction endonuclease *Hin*dIII. The DNA was electrophoresed on a 0.8% agarose gel, transferred to nylon membrane and probed with [^{32}P]-labelled *Opaque-2* cDNA amplified by PCR. *Lanes 1* and *16* 1-kb (BRL) DNA marker, *lanes 2–7* BC population (TX5855/POB67 × POB67), *lanes 8–15* BC population (TX5855/POB67 × TX5855). *Lane 8* TX5855 (*O2/O2*), *lane 9* homozygous recessive *opaque-2 (o2/o2)*. Genotypes derived from these results and progeny seed phenotypes are shown in Table 2 for each plant

DNA probe

A full length *Opaque-2* cDNA clone, pMM1a, was obtained as a gift from M. Maddaloni (Bergamo, Italy). To maximize the specificity of the probe, primers corresponding to an internal region of the *O2* gene extending from the 3′ end of exon 1 (*O2*-3 primer sequence: GTACAATGCCATACTGAGGAG) to the 5′ end of exon 6 (*O2*-5 primer sequence: CCACTATCTCTACTCGAG) (Maddaloni et al. 1989) were used to amplify a fragment by polymerase chain reaction (PCR) (Mullis et al. 1986; Mullis and Faloona 1987). PCR reactions were carried out in an M J Research Programmable Thermal Controller using Taq DNA polymerase and buffers provided by Promega Biotech, Madison, Wis. The parameters for PCR amplification were as follows: 1 min at 90 °C, 2 min at 45 °C, 2 min at 72 °C for 40 cycles followed by a 10 min incubation at 72 °C. PCR products were electrophoresed on 1.5% low-melting point (LMP) agarose, and the desired band was isolated. The band was spin filter purified and radiolabelled with α-[^{32}P]dATP using random primer labelling (Feinberg and Vogelstein 1983).

Hybridization

Established protocols for hybridization and washing (Sambrook et al. 1989; Church and Gilbert 1984) were used with some modification (Taylor et al. 1993). The hybridization buffer consisted of 0.25 *M* $NaHPO_4$ (0.125 m Na_2HPO_4 titrated to pH 7.2 with H_3PO_4), 2.5 m*M* EDTA, 7% sodium dodecyl sulfate (SDS), 1% nonfat milk, and 5% polyethylene glycol (PEG). Membranes were sealed in a heat-sealable bag along with the hybridization buffer, prehybridized for 1–2 h at 65 °C, and later hybridized to 50 ng of denatured *O2* probe at 65 °C for 24 h. The membranes were removed from the bag and washed in 0.25 *M* $NaHPO_4$ (pH 7.2), 2% SDS, 1 m*M* EDTA for 2×30 min at 65 °C, then in 0.04 *M* $NaHPO_4$ (pH 7.2), 1% SDS, 1 m*M* EDTA for 2×30 min at the same temperature. The washed membranes were blotted dry, covered with a plastic wrap and autoradiographed using Kodak XAR-5 film with one intensifying screen (Dupont Lightning Plus) at –80 °C for 1–3 days.

Results

To determine whether polymorphisms could be detected with the *O2* cDNA probe, DNA from a standard inbred

(Mo17) and the same inbred converted to homozygous *opaque-2* was digested with 14 different restriction endonucleases, size separated on an agarose gel, blotted to nylon membrane, and hybridized to a radiolabelled full-length *O2* cDNA. The result is shown in Fig. 1. Of the 14 enzymes tested, 10 showed usable polymorphisms. All of the nonusable enzymes were methylation sensitive.

On the basis of our initial survey, *Hin*dIII was chosen as a standard enzyme for *opaque-2* analysis in the segregating populations. This choice was based on the cost, enzyme stability, and the large difference in the sizes of the bands detected in the *o2/o2* and *O2/O2* lines. The banding patterns for the standard genotypes used in this assay, TX5855 (*O2/O2*), and homozygous recessive mutants (*o2/o2*) were determined using the PCR-amplified *O2* probe and *Hin*dIII restriction digests of genomic DNA. The W64A*o2* genotype (*o2/o2*) was detected as a band of approximately 6.5 kb, while the band for the wild-type genotype (*O2/O2*) was approximately 16.5 kb. Seventy-two F_2 seedlings and 108 seedlings from the reciprocal BC populations were analyzed. The genotype of individual seedlings was scored by the presence or absence of a specific band pattern as compared to the standards. The band patterns illustrated in Fig. 2 are typical of the analyses that were conducted using the *O2* probe to identify genotypes in the F_2 and BC populations. In each case, the genotypes were readily identified.

To establish the accuracy of the genotypic identification of individual seedlings based on the *O2* probe, progeny seed phenotypes of each plant in the F_2 and BC populations were scored by visual examination on a light box. Table 2 shows the correspondence of progeny seed phenotypes with the seedling genotype identified in Figure 2. The seed phenotypes are illustrated in Fig. 3a–d. The range of translucence in these kernels indicates that the opacity in the homozygous *o2* seed varies in different genetic backgrounds.

The summary of the populations analyzed using the RFLP marker assay is shown in Table 3. Of the 72 F_2 seedlings analyzed from different populations, 16 were *O2/O2*, 39 were *O2/o2*, and 17 were *o2/o2*. The ratio of *O2/O2*, *O2/o2*, and *o2/o2* genotypes was 1: 2.4: 1.06. Of the 56 seedlings analyzed from the BC population $F_1 \times o2/o2$ parent, 28 were *O2/o2* and 27 were *o2/o2* genotypes, making the genotypic ratio 1.04: 1.0. One of the seedlings analyzed was identified as *O2/O2* genotype. The progeny seed phenotype showed that the kernels are normal and completely translucent. Since the cross was between *O2/o2* and *o2/o2*, there is no expectation of the *O2/O2* genotype, and this particular seedling appears to be a seed contaminant (pollen contamination is not a rare event in materials that are hand pollinated in the midst of other maize plants). Of the

Table 2 Comparison of juvenile plant genotypes of BC population with progeny seed phenotypes. The juvenile plant genotypes were identified by Southern blot procedure (Fig. 2). Each plant was self pollinated and the ears were scored phenotypically by visual examination of the progeny seeds on a light box (Fig. 3)

Lane number	Seedling genotype	Progeny seed phenotype
2	*O2/o2*	Segregating
3	*o2/o2*	Opaque
4	*O2/o2*	Segregating
5	*O2/o2*	Segregating
6	*O2/o2*	Segregating
7	*O2/o2*	Segregating
10	*O2/o2*	Segregating
11	*O2/O2*	Translucent
12	*O2/o2*	Segregating
13	*O2/o2*	Segregating
14	*O2/O2*	Translucent
15	*O2/o2*	Segregating

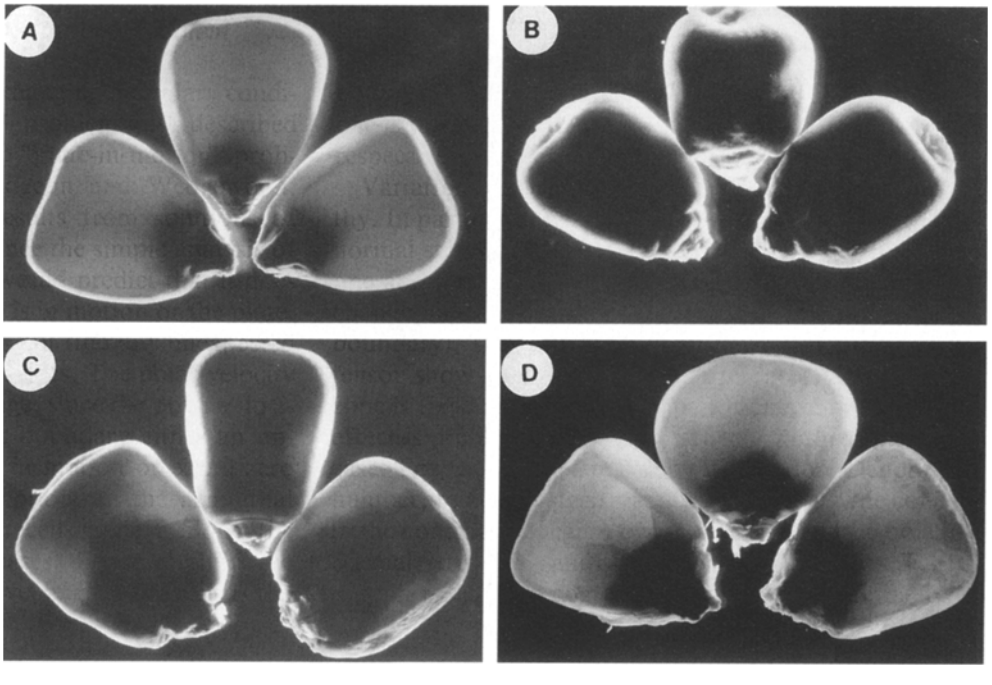

Fig. 3A–D Normal maize (*O2/O2*) and *opaque-2* maize seed phenotypes. **A** Translucent phenotype, **B** opaque phenotype **C** partially opaque phenotype **D** completely translucent opaque phenotype

Table 3 Number of seedlings analyzed in each population and their classification according to different genotypes and the genotypic ratio in each population

Populations	Genotypes			Total number of plants	Observed ratio	Expected ratio	Chi^2 ratio	*P*
	O2/O2	*O2/o2*	*o2/o2*					
F_2 populations								
(TX5855/POP20)×(TX5855/POP20)	6	16	7	29				
(TX5855/POP22)×(TX5855/POP22)	4	9	2	15				
(TX5855/POB67)×(TX5855/POB67)	5	15	8	28				
Total number of plants/genotype	15	40	17	72	1.0:2.6:1.1	1:2:1	0.99	<0.05
BC populations								
(TX5855/POP20)×(POP20)		16	16	32				
(TX5855/POP22)×(POP22)	1[a]	12	11	24				
Total number of plants/genotype	1	28	27	56	1.0:1.04	1:1	0.02	<0.05
BC populations								
(TX5855/POP20)×(TX5855)	8	16		24				
(TX5855/POP22)×(TX5855)	6	8		14				
(TX5855/POB67)×(TX5855)	10	4		14				
Total number of plants/genotype	24	28		52	1.0:1.16	1:1	0.30	<0.05

[a] BC population (F_1×*o2/o2*) should not have any seedlings with *O2/O2* genotype, this plant appears to be a contaminant

52 BC (F_1×*O2/O2* parent) seedlings assayed using the RFLP marker and Southern procedures, 24 seedlings were *O2/O2* and 28 were *O2/o2*, making the genotypic ratio 1.0:1.16 (Table 3). All of the obtained ratios were in accordance with the expected Mendelian ratios for single gene segregation (i.e., 1:2:1 for F_2 and 1:1 for both BC segregating populations).

Discussion

The objective of this study was to develop an *opaque-2* RFLP marker assay and to test its effectiveness in identifying the *O2/O2*, *O2/o2*, and *o2/o2* genotypes in segregating populations. RFLP analysis detects mutations or rearrangements in the genome that alter the distribution of specific endonuclease recognition sites at defined sites in the genome and has been successfully used in plant breeding (reviewed by Murray et al. 1988; Helentjaris et al. 1985; Tanksley et al. 1989; Stuber 1992). Because a probe derived from the *opaque-2* gene would minimize recombination between the target gene locus and RFLP, an RFLP marker assay was developed using the *O2* cDNA as a probe on *Hin*dIII-digested genomic DNA. A number of other enzymes also detected polymorphisms. Field testing showed that this assay was effective for identification of the *opaque-2* genotypes (*O2/O2, O2/o2*, and *o2/o2*) in segregating populations. Specific bands obtained by Southern blot hybridization of the *Hin*dIII-digested genomic DNA from individual seedlings with the *O2* probe correlated with a specific *opaque-2* genotype. A single band of 16.5 kb indicated that the plant analyzed was *O2/O2,* and a single band of 6.5 kb indicated that the plant was *o2/o2*. If two bands, i.e., 16.5 and 6.5 kb, were present the genotype of that individual plant was *O2/o2* (Fig. 2).

Previous methods to differentiate normal and *opaque-2* forms included visual screening, biochemical analysis, and zein electrophoretic and chromatographic profiles (for review, see Villegas et al. 1992; Larkins et al. 1992; Paulis et al. 1992). The *opaque-2* kernels segregating in normal ears of maize usually are sufficiently distinctive to allow them to be identified, but these kernel differences are obscured in the hard endosperm QPM seed to the extent that visual identification of the *o2/o2* ears is not reliable. Visual screening of seed phenotypes in such cases is difficult. The seed phenotypes of *opaque-2* and hard endosperm *opaque-2* kernels shown in Fig. 3b–d indicate that there is variable translucence in different genetic backgrounds, making some of the *o2* phenotypes indistinguishable from their normal counterparts. Integration of RFLP analysis into the selection procedures eliminates the need for testcrossing to confirm the presence of the *o2* allele. Most selection methods previously used (Paulis et al. 1992; Wallace 1989; Larkins et al. 1992) to differentiate normal and *opaque-2* forms are inefficient because the genotypes are identified after the selections are made for the seed and plant characteristics. The analysis of leaf samples of individual juvenile plants by RFLP assay means that the genotypes can be identified before the pollinations are made. Thus, by the incorporation of probe data into selection procedures, simultaneous, rather than sequential, selection of seed and plant characteristics is achieved.

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