Plant Molecular Biology Reporter
Volume 9(4) 1991

pages 294-307

Review

DNA Amplification Fingerprinting: A Strategy for Genome Analysis

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Key Words: DNA fingerprinting, arbitrary primers, PCR, genome mapping

Abstract: A novel strategy to detect genetic differences among organisms, DNA amplification fingerprinting (DAF), uses a thermostable DNA polymerase directed by usually one short (≥5 bp) oligonucleotide primer of arbitrary sequence to amplify short segments of genomic DNA and generate a range of DNA extension products. These products can be analyzed by polyacrylamide gel electrophoresis and silver staining. DAF is rapid and sensitive and is independent of cloning and prior genetic characterization. Here we describe this new methodology, its application to plant genotyping, and its perspectives in DNA fingerprinting and genome mapping.

The characterization of a DNA sample for individual identity according to its chemistry or sequence information, often referred to as DNA "fingerprinting", has been used for genome linkage mapping, identity testing, determination of family relationship and genetic variation, population and pedigree analysis, forensic identification, localization of disease loci, and epidemiology (Watkins, 1988; Donis-Keller et al., 1987; Landegren et al., 1988).

Abbreviations: DAF, DNA amplification fingerprinting; AFLP, amplification fragment length polymorphism; PCR, polymerase chain reaction; cM, centimorgan; STS, sequence-tagged sites; IRS, interspersed repetitive sequences.

In contrast to many techniques for biological comparison, such as blood group typing and isozyme analysis, DNA-based methodologies directly reflect the relatedness or phylogeny of the sample material. Moreover, they can potentially distinguish single cells from an individual, since DNA chemistry varies between cell types and base pair mutations are stably inherited at every cell division cycle. Because of its plasticity, ubiquity and stability, DNA is the ideal milieu of analysis. For example, DNA from minute and relatively degraded forensic samples, DNA material from only a few cells, or ancient DNA from mummified or fossilized material can be analyzed using DNA amplification (Pääbo, 1989; Golenberg et al., 1990; Hagelberg et al., 1991).

Variation in the nucleotide sequence of DNA can be exploited to produce characteristic fingerprints. Often entire genomes must be examined or compared and current effort is to reduce the enormous complexity of the DNA starting material into simple but characteristic patterns.

Conventional DNA fingerprinting. The detection of a polymorphic DNA locus characterized by a number of variable-length restriction fragments, termed restriction fragment length polymorphisms (RFLPs), was originally used to fingerprint genomes (Botstein et al. 1980; Wyman and White, 1980). Following DNA cleavage by restriction enzymes, variations in fragment lengths occur because mutations create or abolish restriction sites in the DNA. To identify these polymorphisms, digests are separated by electrophoresis, transferred to a membrane support, and hybridized to isotopically labeled DNA probes. Fingerprints revealed as variant banding patterns on X-ray films are the result of polymorphic fragments detected by the chosen probe. The probe DNA may hybridize to multiple tandem-repetitive or hypervariable minisatellites (Jeffreys et al., 1985a,b; Vassart et al., 1987) and produce complex fingerprint patterns. Alternatively, probes may be locus-specific for individual hypervariable loci (Wong et al., 1986; Nakamura et al., 1987; Wong et al., 1987; Armour et al., 1989) and produce simpler patterns by detecting alleles from single or even multiple loci.

New approaches to DNA fingerprinting. The enzymatic amplification of specific DNA sequences using the *polymerase chain reaction* (PCR) (Mullis et al., 1987; Saiki et al., 1988; Ochman et al., 1988; Erlich et al., 1991) has been successfully used in conventional DNA fingerprinting. PCR

uses two oligonucleotide primers of about 20 nucleotides in length that specifically hybridize to opposite DNA strands flanking a region to be amplified. A series of cycles involving DNA denaturation, primer annealing and extension of the annealed primers by DNA polymerase, will specifically amplify the target DNA region many million-fold to produce an amplified fragment with its termini defined by the 5' end of each primer.

PCR has been used to incorporate radiolabeled nucleotides into probes for DNA fingerprinting. These probes have a very high specific activity and are especially useful when the ratio of probe to target size is small. The PCR process can also be directed to the specific amplification of hypervariable regions within defined loci (Jeffreys et al., 1988; Boerwinkle et al., 1989; Horn et al., 1990). These "minisatellite" DNA regions consist of motifs 15 to 30 nucleotides long repeated in tandem at various loci and can be used for DNA typing. Short repetitive "simple sequence" motifs, only 2 to 10 nucleotides in length, that are tandemly arranged to form long hypervariable arrays have also been used in a PCR-based assay to detect DNA polymorphisms (Tautz, 1989). Similarly, cocktails of specific primers were used to amplify hypervariable minisatellites from human genomic DNA, and the simple DNA fingerprints obtained were further characterized by internal restriction enzyme analysis (Jeffreys et al., 1990). However, some minisatellites often fail to amplify or produce spurious products.

Changes in DNA sequence and single-base substitutions that induce DNA conformation changes can be detected as shifts in electrophoretic mobility (Orita et al., 1989a). These single-strand conformation polymorphisms (SSCPs) occur in PCR amplified and labeled regions (Orita et al., 1989b) and can identify allelic polymorphism in *Alu* repeats at several chromosomal loci.

DNA amplification fingerprinting using arbitrary primers. Even with the help of PCR, detection of DNA polymorphisms by established fingerprinting procedures requires considerable experimental manipulation and either prior knowledge of the DNA sequence or cloned and characterized probes. To circumvent these requirements, several laboratories have recently used a PCR-based strategy to amplify short arbitrary stretches of DNA from a target genome (Welsh and McClelland, 1990; Williams et al., 1990; Caetano-Anollés et al., 1991). In these studies, amplification using a thermostable DNA polymerase directed by one or

more oligonucleotides of arbitrary sequence generated a characteristic spectrum of products under low stringency conditions.

Welsh and McClelland (1990) produced fingerprints of bacteria and three varieties of rice (*Oriza sativa*) using single arbitrary primers, 20 and 34 nucleotides in length and two cycles of low stringency amplification followed by a series of cycles of high stringency amplification. Polyacrylamide gel electrophoresis and detection by autoradiography resolved between 3 and 20 products. Williams et al. (1990) used shorter arbitrary primers, 9 or 10 nucleotides in length, and low stringency cycles to amplify DNA polymorphisms from several eukaryotic and prokaryotic organisms. When separated by agarose gel electrophoresis and stained with ethidium bromide, up to about 10 products were detected. In this study, polymorphic products were used as genetic markers and several of them were tentatively placed on a RFLP linkage map of soybean. In contrast to the relatively simple fingerprint patterns obtained in these two studies, Caetano-Anollés et al. (1991) used polyacrylamide gel electrophoresis combined with highly sensitive DNA silver staining (Bassam et al., 1991a) to produce detailed and relatively complex DNA fingerprints in a wide variety of organisms. DNA amplification was directed by one or more arbitrary primers as short as five nucleotides in length. This strategy, termed *DNA amplification fingerprinting* (DAF), offers the most advantages. It uses the shortest primers, has the highest resolution and does not require isotopic labeling.

How does DAF work? DAF uses low stringency amplification conditions so that primers can anneal arbitrarily at multiple sites on each template DNA strand. Although initiation of DNA synthesis occurs throughout the template, only those sequences in which priming sites are on opposite strands and in near proximity will be successfully amplified. Mismatch annealing also occurs to a variable extent and can produce less numerous "secondary" amplification products that are also characteristic of the template in study (G. Caetano-Anollés and B.J. Bassam, unpublished).

DAF allows easy detection of DNA polymorphisms that we call *amplification-fragment-length polymorphisms* (AFLPs) following the well established use of the acronym RFLP in conventional fingerprinting. We favor the use of the term AFLP over the term *random-amplified-polymorphic* DNA (RAPD) proposed by Williams et al. (1990) because amplification in DAF is arbitrary but not random. RAPD is also a registered trademark.

Whereas RFLPs detected in conventional DNA fingerprinting result from base-pair changes that alter restriction endonuclease sites within defined loci, AFLPs result from changes in DNA sequence at arbitrary priming sites in the genome. Several mechanisms can account for the variation in length or number of amplification products observed in DAF fingerprints (Fig. 1). AFLPs can arise from nucleotide substitutions that create or abolish primer sites, but also from either the deletion, insertion or inversion of a priming site or of a segment between priming sites. Insertions that separate priming sites rendering them unable to support amplification should also be a source of polymorphisms, as well as any conformational change of the DNA molecules that affect the efficiency of amplification or priming.

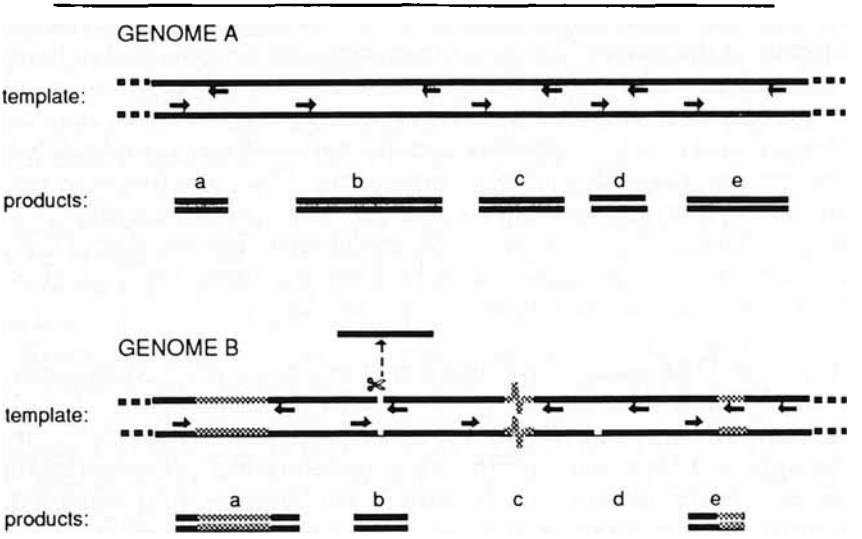

Fig. 1. Origins of amplification-fragment-length polymorphisms. Two related genomes are amplified with DNA polymerase and an arbitrary oligonucleotide primer (arrow) and the resulting amplification products compared. DNA amplification of genome A and B produces five and three DNA fragments respectively. In genome B an insertion between priming sites produces a larger fragment *a*, while a deletion decreases the size of fragment *b*. Additionally, insertion of a large segment of DNA between priming sites eliminates fragment *c* and a point mutation in another priming site eliminates fragment *d*. Finally, an insertion of a DNA segment harboring a priming site in the correct orientation between two existing priming sites decreases the size of fragment *e*. Several other alternatives for the generation of polymorphisms are also possible.

DAF fingerprints have bands that fall into two categories, those that are phylogenetically conserved and those that are individual-specific. This suggests that primer sites are randomly distributed along the target genome and flank both conserved and highly variable regions. There is also wide variation in the degree of amplification between different fragments that is reproducible between experiments. This could result from multiple copies of the amplified regions, reflect the efficiency with which particular regions are amplified or result from mismatch annealing.

Optimization of amplification. DAF illustrates the importance of an appropriate separation/detection procedure to resolve adequately the spectrum of amplified products into a characteristic and reproducible fingerprint pattern. High-resolution separation and staining of DNA was crucial to detect amplification products at the picogram level (Bassam et al., 1991b). If techniques with lower resolution are used, only the few predominant amplification products will be detected and many primers that could give useful fingerprints will produce few or no detectable products or even irreproducible patterns.

Although fingerprints can be easily obtained, several parameters of the amplification reaction need to be optimized to produce reproducible fingerprints. Generally, the concentration of enzyme, template and magnesium and the number of cycles must be optimized. The use of more than 1 ng·μL^{-1} of template and of the Stoffel fragment, an engineered DNA polymerase from *Thermus aquaticus* lacking 289 amino acids from the N-terminal portion and 5′- to 3′-exonuclease activity, were important to generate reproducible fingerprints in bacteria (B.J. Bassam, G. Caetano-Anollés and P.M. Gresshoff, submitted). Some turfgrass material also required careful optimization (K. Weaver, unpublished). Similarly, amplification with unoptimized magnesium concentrations produces variant and irreproducible bacterial fingerprints.

Fingerprint tailoring. Fingerprint patterns produced by DAF can be tailored to suit particular requirements. Generally this is done by selecting an appropriate primer.

Tailoring the number of products. Relatively simple patterns are suitable for genetic mapping, whereas more complex patterns with higher information content are most useful for genotyping. The number of detectable

amplification products can be quite variable, ranging from less than 10 to over a hundred. Whereas larger genomes can produce more products, their number is largely determined by the primer (Caetano-Anollés et al., 1991). In particular, there is some evidence that high GC content of the primer results in more products. Increasing the annealing temperature can also decrease the number of amplified products, and is an example of how amplification conditions can also be used to tailor fingerprints.

Tailoring DNA polymorphisms. The choice of primer also affects the relative numbers of polymorphic vs. monomorphic products. A higher proportion of polymorphic products is most valuable for determining individual identity whereas monomorphic products are useful for characterization of organisms at the species level.

Multiplex DAF. Another way to tailor fingerprints is to use more than one primer and produce new patterns of amplified products. Fingerprints produced with more than one primer are not merely the result of combining the amplification products obtained using separate primers (Caetano-Anollés et al., 1991).

The use of AFLPs in the analysis of a wide range of organisms. DAF has been successfully used to detect genetic differences among animals, plants and bacteria. The detailed fingerprint patterns generated with this methodology can differentiate human (Caetano-Anollés et al., 1991) and coyote (S. MacKenzie, unpublished) individuals. In these studies, amplification with some primers produced monomorphic patterns useful for identification at the species level. In humans, DNA polymorphisms were inherited from either parent, indicating that these AFLPs can be used as genetic markers (Caetano-Anollés et al., 1991). DAF was also used to identify various inbred strains of mouse (*Mus musculus*) and polymorphisms from recombinant homozygous inbreds were used as markers and placed within a genetic linkage map (Welsh et al., 1991).

Prokaryotic organisms can also be fingerprinted. Clinical isolates of *Streptococcus uberis* (B. Jayarao, B. J. Bassam, G. Caetano-Anollés, P. M. Gresshoff and P. Oliver, submitted), *Streptococcus pyogenes,* several species of *Staphylococcus* (Welsh and McClelland, 1990), *Escherichia coli* and *Klebsiella pneumoniae* (B. J. Bassam, G. Caetano-Anollés, and P. M. Gresshoff, submitted) were readily identified. This shows how DAF can be used for clinical purposes in the diagnosis of human and animal pathogens. In particular, restriction endonuclease fingerprinting and

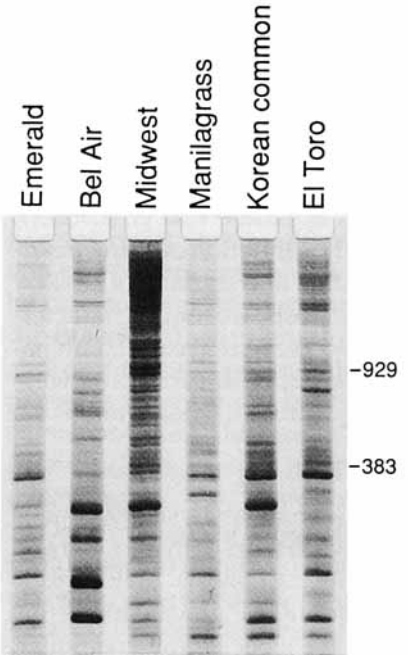

Fig. 2. Polymorphic patterns detected in *Zoysia* species. Amplification was done in a 50 µL-reaction volume with 100 pg of template DNA, 0.5 µg of primer GTGACGTAGG, 2 units of AmpliTaq DNA polymerase (Perkin-Elmer/Cetus), in reaction buffer [10 mM Tris.HCl (pH 8.3), 50 mM KCl and 2.5 mM $MgCl_2$] containing 200 µM of each dNTP (Pharmacia). The reaction mix was overlaid with 2 drops of mineral oil and amplified in an Ericomp thermocycler for 40 cycles (1 sec at 96°C, 10 sec at 30°C, and 10 sec at 72°C, with heating and cooling rates of 23°C·min^{-1} and 14°C·min^{-1} respectively). Amplification products were separated by polyacrylamide gel electrophoresis combined with silver staining (Bassam et al., 1991a). Amplification profiles were obtained with *Zoysia* sp. cv. Emerald , *Z. japonica* cv. Bel Air , *Z. japonica* cv. Midwest, *Z. matrella* cv. Manilagrass, *Z. japonica* cv. Korean common, and *Z. japonica* cv. El Toro. Cultivar Emerald is an F_1 hybrid between *Z. matrella* cv. *Z. japonica* and *Z. matrella* cv. *Z. tenuifolia*.

DAF analysis of *S. uberis* produced comparable groupings of the bacterial isolates examined (B. Jayarao, G. Caetano-Anollés and B. J. Bassam, unpublished). Different isolates of the plant pathogen *Discula* which causes dogwood anthracnose (B. J. Bassam, G. Caetano-Anollés and N. Gerahty, unpublished), and of *Leptosphaeria maculans*, the causal agent of blackleg of crucifers (Goodwin and Annis, 1991 were also fingerprinted extending the use of DAF to the study of filamentous fungi.

Within plants, DAF offers the possibility of identifying cultivars and near-isogenic lines. Different cultivars of several turfgrasses were characterized with this methodology. *Zoysia japonica*, *Eremochloa ophiuroides*, *Cynodon dactylon* and *Buchloe dactyloides* cultivars were easily separated (K. Weaver, G. Caetano-Anollés and B. J. Bassam, unpublished). Figure 2 shows typical profiles of *Zoysia* varieties. Similarly, AFLPs were detected between different soybean (*Glycine max*) cultivars (Caetano-Anollés et al., 1991). Varieties of rice (*Oryza sativa*) and inbred lines of maize (*Zea mays*) were also identified using polyacrylamide gel electro-

phoresis and autoradiography (Welsh and McClelland, 1990; Welsh et al., in press). DAF was used to separate cultivars of dogwood (*Cornus florida*) and differentiate them from other *Cornus* species (B. J. Bassam and G. Caetano-Anollés, unpublished). Cultivars of peanut (*Arachis hypogaea* L.) were not separated but wild *Arachis* species were readily identified when using agarose gel electrophoresis and ethidium bromide staining of amplified DNA (T. Halward, T. Stalker, E. LaRue and G. Kochert, submitted). This probably points towards the limitation of using insufficiently sensitive techniques for the separation and staining of DNA.

In the analysis of the *Azolla-Anabaena* symbiosis, we found characteristic patterns for both the microsymbiont and the fern in each combination, but most importantly the contributions of both symbionts were clearly identified (D. Eskew, G. Caetano-Anollés, B. J. Bassam and P. M. Gresshoff, in preparation).

The use of AFLPs as genetic markers. Genome analysis by genetic linkage is based on DNA recombination between pairs of maternally and paternally derived chromosome homologs during meiosis. Major efforts have been mounted to obtain high-resolution RFLP genetic maps in a number of plant species (Helentjaris et al., 1986; Beckmann and Soller, 1986; Bernatzky and Tanksley, 1986; Landry et al., 1987; Helentjaris et al., 1988; Chang et al., 1988) and in some cases to the resolution of several loci affecting quantitative traits (Paterson et al., 1988; Tanksley and Hewitt, 1988; Keim et al., 1990). RFLP markers can be placed within a linkage map, like the one in soybean, with an average spacing of about 9 centimorgans (cM) (Keim et al., 1990). In humans, genetic linkage can localize a gene-containing region to within 1 to 10 cM with a probability of 95% (Donis-Keller et al., 1987). On average, 1 cM corresponds to 1 Mb of DNA. Since the average number of genes in the human genome is 30 per cM, considerable effort is required to identify a mutant gene within a large chromosomal region. RFLP maps with lower marker density, like that of soybean, pose an even greater challenge. Recently, polymorphic sequences in single sperm cells were amplified and the allelic status of each locus determined by hybridization to allele-specific oligonucleotide probes (Li et al., 1988) or direct examination of PCR products in polyacrylamide gels (Li et al., 1990). This permited the analysis of a large number of individual meiotic products and the study of recombination in haploid cells. This strategy constitutes an alternative to pedigree analysis and could be used to construct high-density genetic maps from male individuals.

Physical mapping techniques complement genetic linkage studies. Generally, physical mapping is done by saturation cosmid cloning. This generates a series of overlaping clones or "contigs," 100 to 200 kb in length, that span a relatively large chromosomal region. Cloning of DNA segments, less than 1 Mb in length, into yeast artificial chromosomes (YAC) (Green and Olsson, 1990) and radiation hybrid mapping of mammalian chromosomes (Cox et al., 1990) assemble stretches of contiguous order information at the 500 kb level of resolution. Direct analysis of larger regions of uncloned genomic DNA requires cleavage by rare-cutter restriction enzymes, separation of large DNA fragments (up to about 20 Mb in length) by pulsed-field gel electrophoresis (PFGE), and ordering of contiguous fragments. Finally, *in-situ* hybridization provides the means of localizing molecular probes to specific chromosomal positions (Lichter et al., 1990). This "chromosome painting" procedure can locate a marker to within 2% of chromosomal length in metaphase.

Development of alternative strategies to increase marker density should permit improved genetic linkage analysis and facilitate physical mapping. The use of "sequence-tagged sites" (STS), a class of landmarks 200 to 500 bp long distributed about 100 kb apart throughout the genome, is seen as the common element necessary to integrate physical and genetic maps (Olsson et al., 1989). These STS will eliminate the need to store and distribute DNA probes, and will establish a database for the design of amplification primers. The amplification of STS within YAC clones and the assemblage of contig maps has been proposed as a strategy for the analysis of large regions of DNA (Green and Olsson, 1990). Interspersed repetitive sequences (IRS) like Alu or Kpn can also serve to amplify DNA fragments that can function as hybridization probes or sequencing templates. IRS-generated markers would act as anchor points to link both genetic and physical maps. Similarly, short repetitive sequences, now shown to be common in plants (Weising et al., 1991), could be used to define STS in a genome.

AFLP-based linkage analysis with very short oligonucleotide primers should be particularly useful for high density mapping. Since DAF is simple and amenable to automation, the information obtained can be easily "translated" to a DNA sequence-based physical map, and could be used not only to generate denser linkage maps but also to define initial anchor sites for further STS characterization.

Using agarose gel electrophoresis and ethidium bromide staining, variant amplification products appear to be either present or absent

(Williams et al., 1990). Based on these results, AFLPs appear to behave as dominant markers limiting its use to inbred lineages. Although, AFLP markers can be co-dominant, we are currently examining whether DAF is quantitative enough to distinguish between homozygotes and heterozygotes. AFLP markers have been used for mapping of inbred lines in soybean (Williams et al., 1990; A. Kolchinsky, unpublished) and mouse (Welsh et al., 1991).

One limitation of physical mapping strategies is obtaining useful markers for each end of cloned DNA fragments allowing identification of overlaps in YAC and cosmid libraries. DAF was used to fingerprint yeast chromosomes and large genomic DNA fragments from soybean isolated by pulsed field gel electrophoresis (A. Kolchinsky and R. Funke, unpublished). This "localized fingerprinting" procedure could determine anchor points important for both genetic and physical mapping.

Future directions. We envision the automation, integration and standardization of all steps in the DAF methodology for the routine fingerprinting analysis of DNA samples and for genome mapping:

- *Automated DNA extraction.* DAF is largely independent of the amount and quality of template DNA minimizing the number of enzymatic and chemical treatments currently required for automated DNA extraction.
- *Robotic manipulation of pre-amplification steps.* DAF utilizes few reagents in few reaction steps, making automation simple and easily linked to the amplification step in the thermocycler.
- *Amplification.* Automated amplification will couple existing thermocycling methodology to input-output devices that will handle sample delivery and the continuous monitoring of products to further optimize the amplification reaction.
- *Post-amplification analysis.* We are currently investigating the use of multi-color fluorescence detection and computer-based, real-time analysis of electrophoretic band patterns (now routinely used for DNA sequencing) to resolve amplification fingerprints quickly and with high accuracy. Other separation procedures that are worth examining, such as capillary electrophoresis, could resolve fingerprint products directly in the liquid phase.

The advantages of DAF over current DNA fingerprinting methodologies have been already addressed (Bassam et al., 1991b). In single short experiments, DNA fingerprints can be produced with greater resolution

and information content than is possible by conventional techniques. Because DAF does not use radiochemicals, photography, or highly sophisticated equipment it can be used in laboratories with limited resources like many in underdeveloped and developing countries. Such a powerful and flexible tool will be valuable for identity testing, population and pedigree analysis, molecular characterization of near isogenic lines and high density genetic mapping.

Acknowledgments. We thank K. Weaver, A. Kolchinsky, R. Funke and S. MacKenzie for sharing results prior to publication and to L. Callahan for providing turfgrass DNA. This work was supported by an endowment to the Racheff Chair of Excellence in Plant Molecular Genetics.

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