Identification of Simple Sequence Repeat Markers that Differentiate Bermudagrass Cultivars Derived from ‘Tifgreen’

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ABSTRACT. The release of the bermudagrass (*Cynodon* spp.) triploid hybrid ‘Tifgreen’ revolutionized southeastern U.S. golf course greens. Off-types within this cultivar began to be identified soon after the initial plantings, and through the last 50 years, many of the best performing off-types have been released as new cultivars. Examination of some of the most popular somatic mutants with a new set of 47 simple sequence repeat (SSR) markers and 23 previously discovered genomic SSR markers identified five polymorphic fragments (as compared with ‘Tifgreen’) among three cultivars, TifEagle, MiniVerde, and Tifdwarf. Each polymorphism appears to be a slight increase/decrease in microsatellite repeat number and the polymorphic fragments are unique for each cultivar. Two polymorphic fragments were identified that were unique to ‘Tifdwarf’, one polymorphic fragment was unique to ‘TifEagle’, and two polymorphic fragments were unique to ‘MiniVerde’. Furthermore, three of the five polymorphic markers display an additional allele only in the shoot tissue but not in the root tissue of ‘TifEagle’ and ‘Tifdwarf’. This finding suggests that ‘TifEagle’ and ‘Tifdwarf’ are somatic chimeras. This set of SSR markers identifies repeatable polymorphic fragments among multiple ‘Tifgreen’-derived cultivars and gives insight into the nature of the mutations that exist within ‘Tifgreen’.

The release of ‘Tifgreen’ bermudagrass in 1956 launched the era of vegetatively propagated turfgrasses using a production method that preserved the initial hybrid vigor of new cultivars barring genetic instability (Hanna and Anderson, 2008). ‘Tifgreen’ originated from a cross between an Egyptian *Cynodon transvaalensis* introduction and a common bermudagrass (*Cynodon dactylon*) from a North Carolina golf course green (Hein, 1961). This cultivar can be mowed daily at a plant height of 4.7 mm, which at the time of its release represented a breakthrough in putting green quality. The ability to uniformly mow ‘Tifgreen’ at lower heights spurred golf courses across the southeast to replace their common bermudagrass greens (Hanna and Anderson, 2008).

Well-defined areas with noticeable differences in plant morphology (i.e., plant color, leaf size, and internode length) were observed on ‘Tifgreen’ putting surfaces soon after its release. Several of these “off-types” were isolated from putting greens in Florence, SC, and Sea Island, GA, and further evaluated at lower mowing heights than ‘Tifgreen’ could tolerate. The term off-type refers to a plant that has a different growth habit, density, texture, and color that disrupts uniformity in monoculture plantings (Capo-chichi et al., 2005). The off-type

selected from Florence Country Club was released as ‘Tifdwarf’ (Burton, 1966; Elsner, 1966) and became one of the most widely used grasses on greens in the warmer regions of the world.

Off-types found in ‘Tifgreen’ or ‘Tifdwarf’ make up the latest generation of bermudagrass for putting greens. These cultivars include Champion, Classic Dwarf, FloraDwarf, Jenson, Jones Dwarf, MiniVerde, MS Supreme, Pee Dee 102, Quality, Reesegrass, and TifEagle (Harris-Shultz et al., 2010; McCarty and Canegallo, 2005). ‘Champion’, ‘FloraDwarf’, ‘Jones Dwarf’, ‘MiniVerde’, ‘MS Supreme’, ‘Tifdwarf’, and ‘TifEagle’ were highly genetically similar to ‘Tifgreen’ when DNA fingerprinting was used and appear to be somatic mutants (Capo-chichi et al., 2005; Harris-Shultz et al., 2010; Wang et al., 2010). Although many cultivars have been derived from ‘Tifgreen’ and ‘Tifdwarf’, most mutations are deleterious. Inconsistencies in appearance, playability, response to environmental conditions (i.e., nutrient and water availability), herbicide application, etc., have resulted in severe problems and millions of dollars in loss to the golf course industry and sod farms (Caetano-Anolles et al., 1997).

Off-types in bermudagrass can be generated by either clonal variation or by contamination from an unrelated bermudagrass through seed germination or sprig introduction. Clonal variation can be the result of epigenetic modification in response to the environment, from the presence of plant pathogens, or, more commonly, from mutations that occur during growth (Pelsy, 2010). Genetic mutations can be induced by chemicals such as herbicides that affect microtubule formation during

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mitosis (Capo-chichi et al., 2005) or can occur at random by a natural process such as transposon activity and problems with DNA repair (Leroy and Leon, 2000; Wessler, 2001). A mutation in one cell of a layer of the shoot apical meristem increases by mitosis and produces a mutated sector (Hocquigny et al., 2004). These plants are periclinal chimeras because one or two entire cell layers of the apical meristem are genetically distinct from the adjacent layers. Periclinal chimeras are often stable and can be maintained by vegetative propagation of stems (Dermen, 1960) and are common in long-lived clonally propagated crops (Franks et al., 2002).

Grapes (*Vitis vinifera*), like bermudagrass, have cultivars that are maintained by vegetative propagation to create clones that are genetically identical to the parent plant assuming that no somatic mutation occurred in the regenerating cells that gave rise to the clone, yet similar to bermudagrass, grape clones as well as many species propagated vegetatively for commercial horticulture often display phenotypic variation (Gill et al., 1995; Pelsy, 2010). When microsatellite markers were used among clones from many grape cultivars (which are diploids), three to four alleles were detected (Franks et al., 2002; Hocquigny et al., 2004). This increase in allele number was the result of expansion or contraction of the repeat motif in a cell layer of grape forming a periclinal chimera (Hocquigny et al., 2004). When the cell layers of the periclinal chimera 'Pinot Meunier' were separated by somatic embryogenesis, the regenerated plants not only had distinct genotypes, but had novel phenotypes as compared with the parental plant (Franks et al., 2002). It has been noted that it is easy to miss instances of microsatellite chimerism because minor peaks/bands are often generated (Franks et al., 2002). The genotypes of the cell layers in grape have been determined by studying the roots and sexual progeny because both are descended from the L2 inner tissues and the leaf is derived from L1 and L2 of the plant meristem (Thompson and Olmo, 1963). To our knowledge, the number of cell layers in the tunica of the shoot apical meristem of bermudagrass has not been determined. In monocots, the numbers of tunica cell layers vary from one to four with one and two predominating (Khurana et al., 2004). For example, the shoot apex of wheat (*Triticum aestivum*) consists of both the L1 and L2 cell layers (Simmonds, 1997), whereas the shoot apex of maize (*Zea mays*) is composed of one layer of L1-derived cells (Jackson and Hake, 1997).

The close genetic similarity of cultivars within the 'Tifgreen' family has made it very difficult to distinguish each cultivar from one another using morphological and DNA fingerprinting methods (Harris-Shultz et al., 2010). This creates problems in the protection of cultivar proprietary rights and plant stock certification. The objectives of this study were to identify SSR markers that can distinguish between genotypes in the 'Tifgreen' family and to characterize these polymorphisms in each 'Tifgreen'-derived cultivar.

Material and Methods

PLANT MATERIAL. Nodes from bermudagrass cultivars Jones Dwarf, MiniVerde, Tifdwarf, TifEagle, Tifgreen, TifSport, Tifway, TifGrand, and Tifway II were obtained from B. Schwartz (University of Georgia, Tifton, GA). 'FloraDwarf' and 'Champion' were obtained from the University of Florida Plant Science Research and Education Unit (Citra, FL). Two off-types (MiniVerde T. Lowe 1, MiniVerde T. Lowe 2) and a control

(MiniVerde T. Lowe-Texas) of 'MiniVerde' were donated by T. Lowe (U.S. Golf Assn., Far Hills, NJ). Baytree National Golf Course (Melbourne, FL) donated four samples, three of which are related to 'Tifdwarf'. Many plugs of certified 'Tifdwarf' were collected at Pike Creek Turf (Adel, GA).

PLOIDY ANALYSIS. Ploidy levels were confirmed for each 'Tifgreen'-derived cultivar sample using flow cytometry. Fresh tissue ($\approx 0.5 \text{ cm}^2$) was isolated from potted accessions and chopped using a double-edged razor in 600 μL of nuclei extraction buffer solution (Partec, Munster, Germany) to release the nuclei. The slurry was poured through a 50- μm filter and 1.6 mL of Partec diamidino phenylindole staining buffer was added. The nuclei were analyzed on a Partec Cell Analyzer PAS-III flow cytometer and at least 5000 fluorescent particles were counted. Triploid ploidy level was confirmed by chopping together 'Tifgreen' and a 'Tifgreen'-derived cultivar and confirming that a single Gap1 (G_1) peak was generated.

DNA EXTRACTION AND SIMPLE SEQUENCE REPEAT GENERATION. DNA was extracted from roots or shoots of plants collected as propagules, which were collected from the field or golf course and moved to a glasshouse to accumulate tissue. For extraction of DNA from shoots, shoot tissue was cut into small pieces and placed into 2.0-mL microcentrifuge tubes containing three zinc-plated BBs (Daisy Outdoor Products, Rogers, AR). Samples were flash-frozen in liquid nitrogen, ground using a vortex mixer, and then returned to liquid nitrogen to prevent the tissue from thawing. For extraction of DNA from roots, plants were grown in sand or soil and roots were removed that were healthy and white. These roots were then rinsed with water until the sand or soil was separated and were then frozen and ground using the same method as shoot tissue. A PureLink DNA Purification Kit (Invitrogen, Carlsbad, CA) was used to extract DNA from roots and shoots.

From the tetraploid genotype T89, 143 expressed sequence tag (EST) sequences containing SSRs were identified but not verified by polymerase chain reaction (PCR) (Kim et al., 2008). Primers were designed using Primer 3 (Rozen and Skaletsky, 2000) to flank the SSR sequence from 98 of these ESTs (Harris-Shultz et al., 2010; Table 1, "ES" accessions). Primers used to amplify 25 genomic SSR markers from bermudagrass were previously reported (Harris et al., 2010; Kamps et al., 2011). Chase primers can be found in Kamps et al. (2011). SSRs were amplified and fluorescently labeled for detection using a modified M13-tailed primer method (Boutin-Ganache et al., 2001). PCR reactions were in a 10- μL volume and contained 2 μL of 5 $\times$ Clear GoTaq $\text{\textcircled{R}}$ reaction buffer (Promega, Madison, WI), 1 μL of 25 mM MgCl_2 , 0.8 μL of 2.5 mM dNTP mix, 1.0 μL of pooled M13-tagged forward primer and reverse primer at 1 μM , 1.0 μL of 1 μM M13 primer (M13-TGTAAAACGACGGCCAGT) fluorescently labeled with the IRDye $\text{\textcircled{R}}$ 800 CW fluorophore (Eurofins MWG Operon, Huntsville, AL), 0.04 μL of GoTaq $\text{\textcircled{R}}$ DNA polymerase (Promega), 3.16 μL of sterile water, and 1 μL of 2.5 ng- μL^{-1} DNA. Thermocycler conditions were an initial denaturation at 94 $^{\circ}\text{C}$ for 3 min, 39 cycles of 94 $^{\circ}\text{C}$ for 30 s, 45 to 60 $^{\circ}\text{C}$ (see Table 1) for 1 min, 72 $^{\circ}\text{C}$ for 1 min 10 s, and a final elongation step at 72 $^{\circ}\text{C}$ for 10 min. The thermocycler used was a Gene Amp PCR System 9700 dual block (Applied Biosystems, Foster City, CA). Two microliters of PCR product were combined with 5 μL of Blue Stop (LI-COR $\text{\textcircled{R}}$ Biosciences, Lincoln, NE) and 0.35 μL of this mixture was loaded on a 6.5% acrylamide gel using a LI-COR $\text{\textcircled{R}}$ Biosciences 4300 DNA Analyzer. Gel images were scored visually.

Table 1. Simple sequence repeat markers developed from bermudagrass expressed sequence tags or genomic DNA for this study.

Accession/repeat	Primer-forward	Primer-reverse	Ta (°C) ^z	Size (bp) ^y
ES293131 (TC)15	CGTGATCCTTCCTCCTCTCC	CTACCACAGGCTCCTTGTC	50	125
ES295626 (CT)18,(AT)4	ATGAACCCACCAGCATCTTC	CTGCAAAGGAAACGACAACA	50	190
ES295634 (TC)13	AAGTGCTTGCGGTATGTGTG	CGGCAAGATCCATCCTGTAA	— ^x	153
ES295668 (ACA)6	GCCATGAAGGAATGGAGATG	CCAGCTTGCTTGGGATTAATA	50	156
ES295753 (TTG)10	CCATGTTCTCACTGAAACG	GCGATCAAGGTGAGATACCC	—	243
ES296252 (AG)12	CAGCAACTTCGTCTCGTCAC	GAAGAGGGAGGTGGTGGAG	50	174
ES297067 (TTA)6	GCCGCTCTGTGAATATGCTT	TAACACCACAACGACCTTCG	50	136
ES297215 (CT)4,(CT)10	GGCTCTCTCCCTCTCACACA	CCAGCTCAGCTACATGACCA	50	266
ES297380 (AAC)8,(CT)4	ACCTCTTTGGATTGGTGCAA	TCTTCTAAGCCACATCAAGG	—	278
ES297613 (TAG)7	AAGTGGTCAGGATTTCAACGA	GCAATTACGCAATGGACAAA	50	295
ES298449 (TCTT)11	CTGCTCCGTTTTTGGATAGG	GGGCTTCCGGCAAGTATTTA	50	157
ES298476 (AGT)37	TCATTGAGCATGCAGTACCA	ACCCTGCTTTCCTTTGGATT	50	241
ES298613 (TC)12	AGAGAGCCAACGCCTTCTC	GCACCCATCTCCAGCAGT	50	139
ES299058 (GCC)6,(CTA)7	GAGGCATGTCGCTTCTTCTT	CTGTTGGCACGAATGAATGT	50	125
ES299693 (CGC)7	TACAGCAGCAGAGCAGCAAG	CATCTTCTCCTCCTTGTCG	50	168
ES300003 (CTC)8,(GCA)5	ATGCATTCCAGACTCCATCC	GCCGCCAGAGATGAATACAC	45	227
ES300073 (AGA)6,(GCT)4	AGGCACTGCCAGTTGTAGT	GGGACTGGTTTCACTGCTTG	—	242
ES300208 (TCC)7	GTTGGAGCCAGATCCAGTGT	GCCCAATTGCTTGCTTGAG	50	185
ES300469 (CTCTC)4	CGGAATCGACCTCAACAAGA	GACCATGCCTTAGCCTTAGC	50	134
ES300504 (AGA)7	CTGATGGTGATGCAATGGAC	ACATTTTCTGCTCCTGTGC	—	139
ES300599 (CAG)7	GTAAGCAGTCGCAACCACAG	CATACTGCCCACCCATCTG	—	153
ES301949 (GA)11	CGGTGAGAAGGAGATCGAGA	CCTGCTGTGCCATTGTATTG	50	127
ES302078 (AAAG)5	CATTTTCCCGTATGCTTCTATG	GGTGATGCATAGTCCAGTGGT	50	272
ES302387 (CTC)6	ACTTCGTCATCCCCAGTCAC	CGCCTGGTCTTCTGTATCCT	50	201
ES302424 (AAC)8	CTTTGGATTGGTGCAAGAGG	TCCCTAGCCTACCCCAACTT	50	173
ES302587 (CCG)4	GCCGTACCACTGACCGATTA	CATCGTCCACAACCCAATC	50	150
ES302722 (GC)4,(CCG)7	CCAAGCATACAACCCGTACA	TACCGACGCCGAAGACATA	50	193
ES303244 (CAC)4, (CTG)4,(CCA)6	CTCCTGAGGGGTCTCACTG	CGGAGTAGGTGCTGCTGATT	50	194
ES303320 (TGTTT)4	GATGCGGCAATGTGTTCTTA	ATGGCCAAAACCGTAGTACA	50	156
ES303635 (CT)18,(CA)4	CCGGCAGTCTTCACTTCACT	ACCATCGCAAGAACCAAGAC	50	216
ES303795 (TTG)7	CCCAACTTGCTTGGGATAAA	GAGGTCAAACGACATGATGAA	50	138
ES303825 (AG)10	CCTTGCTCGATCTGGATTTC	CAGCAGCATCTTTGACCTGA	50	156
ES303889 (CGG)7	CACGCTGCTAGGGTTCAATC	GGGAACCGATAATCACTCCA	50	161
ES304692 (TC)7,(CTA)7	CAGATCCATGCTGTTGCTTG	CGGACAATGTTGATCACAGC	50	218
ES304863 (TTA)7	GATTGCTTCGGTAGCTTTGG	CTCGTTAATCTCCCTCTCTCTC	50	147
ES304906 (GAT)7	CAGAACTTCCGCAACATCG	TATGGATCCACCCATAACG	50	172
ES304968 (TC)10	AACACACTCTCGAACCAGACC	CCTGAGATGTGGCAACAGTG	45	123
ES305047 (GAA)7	CGCGCTATCTCCACAACC	GTCTCCATGCGTGAGGTGTA	50	144
ES305124 (TTA)7	GGCAACAGCATTGGTAGACA	GCCAACTTGCAGATTCCAT	50	204
ES305412 (CCT)7	AAGGAAGAAAGCCGAGCAG	GCAGCTTTTCTTGGAATGG	—	263
ES305529 (CT)14	CGTCATAGGTGAGTCGCTTCT	GTCGAGATGAAAGCGGGATA	50	151
ES305668 (TTTG)5	AACGTCCATCCTCTCATCCA	TCGAGTTCGAGTCGAGTTGT	50	93
ES306578 (AG)9(GA)7	GGGGACTCATTCCTCTCTCC	CAGAGCAGGTGATGGTGTG	—	141
ES306759 (CCT)5,(GTC)4	CTCGATAGGCACAAGGGAAG	CCTTGATATCGGAGTCTCTG	50	249
ES306764 (CT)11,(TTC)4	CAGGAACGTGAAGTGACGAG	AGAGGAATCGCACGTACCC	—	129
GU170368 (CTT)10	GAGGCCTCTTGGTGGATAAA	AGCTCGCTCTTGAGCTATGG	—	128
GU170366 (GA)4,(TA)4	GGGGAGGAATGCTAGAAATGA	AGGGCTAGTGGAAGTCCAAA	—	165

^zAnnealing temperature.^yExpected size with M13 tag added to the forward primer.^xNo or poor amplification.

IDENTIFICATION OF POLYMORPHISMS. SSR markers that generated polymorphic bands between ‘Tifgreen’ and the ‘Tifgreen’-derived cultivars were first repeated at least twice using the same genomic DNA from shoots. The cultivars that consistently generated polymorphic fragments with the use of a SSR marker were then sampled from at least six areas of a breeders plot for ‘TifEagle’ (TifEagle A–F) and two areas of a ‘TifEagle’

green (WR-A, WR-B, Green #12; Robins Air Force Base Golf Course, Warner Robins, GA), five areas of a test field for ‘MiniVerde’ (MiniVerde 1–4, 7), two pots of ‘MiniVerde’ grown in sand, two areas of a breeders plot (Tifdwarf breeders A–B), three areas of a test field planted from a foundation field (Tifdwarf 2–4), three areas of certified ‘Tifdwarf’ (Pike Creek 2–4; Pike Creek Turf, Adel, GA), and one plug from

a foundation field of ‘Tifdwarf’ (Tifdwarf Fdn). A separate DNA extraction was done for each area of the plot or field. Foundation stock was unable to be obtained for ‘Jones Dwarf’. Three separate DNA extractions were performed from two pots (Jones Dwarf A–C) of ‘Jones Dwarf’ provided by Dr. Brian Schwartz (Tifton, GA). Consistent amplification of the polymorphic fragment from each area sampled was necessary for ‘Tifgreen’-derived cultivar SSR identification.

CLONING OF CHASE 109 FRAGMENTS. Amplicons for primer pair Chase 109 were amplified from ‘TifEagle’, ‘Tifgreen’, and ‘Tifdwarf’ using the previously described reaction and thermocycler conditions except 5× Green Go Taq Flexi Buffer (Promega) were used and the M13-labeled fluorophore was excluded from the reaction. The ≈300-bp fragments from each genotype were excised from a 1% agarose gel. The fragments were PCR-purified with the Wizard SV Gel and PCR Clean Up System (Promega), ligated into a pGEM T-Easy Vector System (Promega), and transformed in *Turbocells* Chemically Competent *Escherichia coli* (Genlantis, San Diego, CA). Transformed *E. coli* were plated using blue/white selection and white colonies were selected and grown overnight in Luria Broth with 66 µg·mL⁻¹ of ampicillin. Plasmids were purified using a PureYield Plasmid Miniprep System (Promega) and digested with *EcoRI* (Promega) to confirm the presence of an insert. Multiple clones from each genotype were then sequenced at the Laboratory for Genome Technology (Texas A&M University, College Station, TX).

Results and Discussion

IDENTIFICATION OF POLYMORPHIC MARKERS. Previous work using 53 EST-SSR primer pairs provided no markers that could

Table 2. Bermudagrass amplified polymorphic fragments unique to ‘Tifgreen’-derived cultivars.

Cultivar	Primer	Approximate size (bp)	Allele no. change	Shoot/root ^z
TifEagle	Chase 109	308	Yes	Shoot
MiniVerde	ES304692	252	No	Shoot, root
MiniVerde	ES304863	181	No	Shoot, root
Tifdwarf	Chase 33	242	Yes	Shoot
Tifdwarf	Chase 89	250	Yes	Shoot

^zShoot or root refers to whether the polymorphic fragment can be generated in each tissue type.

identify polymorphisms among ‘Tifgreen’ and its derived cultivars (Harris-Shultz et al., 2010). To identify discerning markers, primers to 45 additional EST-SSR sequences and two genomic SSR sequences that were discovered from a bermudagrass resistance gene analog study (Harris et al., 2010) were developed (Table 1). Twenty-three primer pairs from a bermudagrass genomic library (Kamps et al., 2011) were also used to identify polymorphic fragments among the ‘Tifgreen’-derived cultivars, which were confirmed as triploids by flow cytometry (data not shown). From more than 225 scored amplicons, five different sets of primer pairs consistently generated polymorphic fragments among the ‘Tifgreen’-derived cultivars (Table 2). Primer pair Chase 109, the first SSR marker generated to identify ‘TifEagle’ (Kamps et al., 2011) from other ‘Tifgreen’-derived cultivars or ‘Tifgreen’, also generated an additional allele in our study, but at ≈308 bp instead of 450 bp (Fig. 1). Primer pair ES304863 amplified a fragment at ≈181 bp poorly in all ‘MiniVerde’ samples and instead amplified a lower fragment at ≈175 bp as compared with other ‘Tifgreen’-derived cultivars or ‘Tifgreen’ (Fig. 2). Primer pairs Chase 89 and Chase 33 generated an additional fragment in all ‘Tifdwarf’ fragments at ≈250 and 242 bp, respectively (Figs. 3 and 4). The fragments generated by these two sets of primer pairs are very similar in all samples amplified and likely amplify the same locus. Primer pair ES304692 generated an additional fragment in all ‘MiniVerde’ samples at ≈252 bp as compared with ‘Tifgreen’-derived cultivars or ‘Tifgreen’ (Fig. 5). Of interest, two of the five primers (Chase 89, and Chase 33) generated SSR fragments that are actively changing (Figs. 3 and 4). The polymorphic fragment in ‘Tifdwarf’ generated by using primer pairs Chase 89 and 33 appears to be increasing in Tifdwarf breeders B and Tifdwarf 3 and 4. The discovery of an actively mutating locus in ‘Tifdwarf’ agrees with the continual identification of mutations or off-types from this cultivar. Furthermore, collection of certified ‘Tifdwarf’ from Pike Creek Turf Farms (Adel, GA) showed only one additional allele as compared with ‘Tifgreen’ and other ‘Tifgreen’-derived cultivars. Thus, the mutation is specific to the ‘Tifdwarf’ located in Tifton, GA. Interestingly the cultivars Champion, Jones Dwarf, and MiniVerde reported as off-types in ‘Tifdwarf’ greens (Brown et al., 1997; Clemson University, 2009; Dudeck and Murdoch, 1998) do not contain the Chase 89 or a Chase 33 additional ‘Tifdwarf’ allele. This suggests that the Chase 89 or

Fig. 1. Identification of an amplified polymorphic fragment unique to bermudagrass cultivar TifEagle with primer pair Chase 109 using DNA from shoots. Molecular weight (MW) 50- to 350-bp sizing standard (LI-COR Biosciences, Lincoln, NE). BT-2, BT-3, and BT-4 are three samples of ‘Tifdwarf’ from Baytree Golf Course (Melbourne, FL); BT-1 is a contaminant from BayTree Golf Course. ‘TifEagle’ WR-A and WR-B are two samples of ‘TifEagle’ from Robins Air Force Base (Warner Robins, GA). Arrow marks polymorphic fragment present in ‘TifEagle’ samples.

Fig. 2. Identification of an amplified polymorphic fragment absent in bermudagrass cultivar MiniVerde with primer pair ES304863 using DNA from shoots. Molecular weight (MW) 50- to 350-bp sizing standard (LI-COR Biosciences, Lincoln, NE). BT-1 is a contaminant from BayTree Golf Course (Melbourne, FL). Arrow marks polymorphic fragment missing in 'MiniVerde' samples compared with 'Tifgreen' but a slightly lower allele at ≈ 175 bp is present in 'MiniVerde' samples.

Fig. 3. Identification of an amplified polymorphic fragment mutating in bermudagrass cultivar Tifdwarf with primer pair Chase 89 using DNA from shoots. Molecular weight (MW) 50- to 350-bp sizing standard (LI-COR Biosciences, Lincoln, NE). 'Tifdwarf' 2 to 4 were samples taken from a test field planted from foundation material (Tifton, GA). Arrow marks a polymorphic fragment present in 'Tifdwarf'.

Chase 33 mutation in 'Tifdwarf' occurred after the mutations that developed 'Champion', 'Jones Dwarf', or 'MiniVerde'. Alternatively, each ultradwarf cultivar could have had a reversion event, although this is unlikely given that 'Tifdwarf' is actively mutating at this locus in foundation and breeders material located in Tifton, GA. The use of the Chase 89 and Chase 33 amplified markers to identify 'Tifdwarf' in future years may be limited as 'Tifdwarf' changes. Furthermore, the sequences from which the five polymorphic markers were generated have no significant BLAST (blastn, blastx, tblastx)

homology to any sequence in the National Center for Biotechnology Information database.

TEST FOR CHIMERISM. The genotypes of the cell layers from 'Tifgreen'-derived cultivars were explored by isolating DNA from roots on discovery of the addition of at least an allele in 'Tifdwarf' and 'TifEagle' when select microsatellites were amplified (Table 2). Use of the microsatellite markers Chase 89 and Chase 33 failed to generate an additional allele in 'Tifdwarf' root samples as compared with 'Tifdwarf' shoot samples (Fig. 6). Similarly, use of the microsatellite marker Chase 109 failed to generate an additional allele in 'TifEagle' roots as compared with 'Tifdwarf' shoot samples (Fig. 6). Thus, the unique alleles generated from 'Tifdwarf' and 'TifEagle' shoot samples using these primers were not present in the cell layer that formed the roots. This is similar to what was found in grape 'Pinot' clones in which an additional allele was seen in the leaves of clone PG52 but not in the roots at two loci (Hocquigny et al., 2004). Use of the microsatellite markers ES304863 and ES304692 generated the same polymorphic fragment in roots and shoots of 'MiniVerde' samples. Thus, the polymorphic fragments generated from these two primer pairs are present in all cell layers examined (Fig. 7). The occurrence of polymorphic fragments based on tissue type suggests that 'TifEagle' and 'Tifdwarf' are somatic chimeras. This suggests that the 'Tifgreen'-derived cultivars were generated by differential mutation accumulations and subsequent cell layer rearrangements. Phenotypic instability of chimeric plants can be the result of cellular rearrangements in the chimera (Pelsy, 2010). An invasion of cells from the inner layers to the outer layer can occur after meristem damage from herbivores, thus uncovering muta-

tions previously isolated to one cell layer (Marcotrigiano, 2000). Extended periods of low mowing of 'Tifgreen' and its derived cultivars could cause damage to the meristem, thus uncovering mutations that were isolated to one cell layer.

All polymorphisms generated among the cultivars appear to be the result of a slight increase or decrease in SSR repeat number. Indeed, cloning and sequencing of fragments amplified with primer pair Chase109 from 'Tifdwarf', 'Tifgreen', and 'TifEagle' showed expansion and contraction of a long (CA) repeat (Genbank accessions HQ423301-HQ423305).

Continuous vegetative propagation of 'Tifgreen' since the 1950s may have led to the accumulation of mutations, which have formed the 'Tifgreen'-derived cultivars. Alternatively, microsatellite instability, as shown by repeat length polymorphism, is a sign of mismatch repair system deficiency (Leonard et al., 2003). Indeed, addition or deletion of nucleotides may be more frequent where nucleotides are repeated giving rise to slip-mispairing as compared with un-repeated nucleotides; yet the mismatch repair system corrects a large portion of these errors reducing the error rate to below non-repetitive sequences (Leonard et al., 2003). The high mutation rate in these SSR markers may be the result of problems with the mismatch repair system in 'Tifgreen' and its derived cultivars. In contrast, no

polymorphic fragments were identified among 'Tifway', 'Tifway II', and 'TifSport'. 'Tifway II' was created by gamma irradiation of 'Tifway' (Burton, 1985) and 'TifSport' was likely created by gamma irradiation of 'Tifway' or 'Tifway II' (Harris-Shultz et al., 2010). Many more markers are needed to identify fragments that are polymorphic among the 'Tifway'-derived cultivars.

Examination of these five polymorphisms shows that each mutation identified in the derived cultivars is unique and not present in all derived cultivars. Thus, each locus identified has mutated, or is mutating, only in its identified cultivar. Furthermore, the discovery of two loci (ES304863, ES304692) that have mutated in 'MiniVerde' along with the mutations found in 'Tifdwarf' and other cultivars using a relatively small number of fragments suggests that multiple mutations in many loci have developed in the derived cultivars. Mutations such as an expansion or contraction of an SSR not in a multiple of three in a coding region will disrupt the reading frame and may lead to phenotypic changes (Li et al., 2004).

The mutation problem with 'Tifgreen' has been discussed for over 40 years and has been the subject of dozens of scientific papers. This study identified a collection of SSR markers to distinguish between multiple 'Tifgreen'-derived cultivars and gives insight into the nature of mutations in 'Tifgreen' and its derived cultivars.

Fig. 4. Identification of an amplified polymorphic fragment mutating in bermudagrass cultivar Tifdwarf with primer pair Chase 33 using DNA from shoots. Molecular weight (MW) 50- to 350-bp sizing standard (LI-COR Biosciences, Lincoln, NE) 'Tifdwarf' 2 to 4 are samples from a test field planted from a foundation plot (Tifton, GA). Arrow marks a polymorphic fragment present in 'Tifdwarf'.

Fig. 5. Identification of an amplified polymorphic fragment present in bermudagrass cultivar MiniVerde with primer pair ES304692 using DNA from shoots. Molecular weight (MW) 50- to 350-bp sizing standard (LI-COR Biosciences, Lincoln, NE). 'Tifdwarf' 2 to 4 are samples from a foundation field (Tifton, GA). BT-1 is a contaminant from BayTree Golf Course, (Melbourne, FL). Arrow marks a polymorphic fragment present in 'MiniVerde'.

Fig. 6. Identification of an amplified polymorphic fragment present in shoots from bermudagrass cultivars Tifdwarf (Chase 33 and Chase 89) and TifEagle (Chase 109) but not present in roots. Molecular weight (MW) 50- to 350-bp sizing standard (LI-COR Biosciences, Lincoln, NE). Arrows point to the additional fragment; NTC = no template control. Pike Creek samples came from Pike Creek Turf (Adel, GA).

Fig. 7. Identification of an amplified polymorphic fragment present in both shoots and roots from bermudagrass cultivar MiniVerde when primer pair ES304692 (left image) and ES304863 (right image) are used. Molecular weight (MW) 50- to 350-bp sizing standard (LI-COR Biosciences, Lincoln, NE). Arrows point to the alternate alleles.

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