Identification of a locus characteristic of male individuals of buffalo grass [*Buchloe dactyloides* (Nutt.) Engelm.] by using an RAPD marker

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ABSTRACT. Buffalo grass [*Buchloe dactyloides* (Nutt.) Engelm.] plants can be either male, female, or hermaphrodite (monoecious). As there is no morphological difference in the early vegetative growth of these three classes of plants, it is worthwhile to use molecular biological methods to attempt to identify the sex of a plant at this early growth period. In this study, we identified 23 plants that had a stable sex for over at least 3 years. Of these, 9 were male plants, 10 were female plants, and 4 were hermaphrodites. Screening of 300 RAPD primers identified a primer, namely S211 (5'-ttccccgcga-3'), which is capable of identifying male plants. The specific fragment was cloned, sequenced, and submitted to the GenBank database

(accession No. JN982469). When used to identify the sex of 188 plants during their first growing season, the S211 primer correctly identified 85.8% of all male plants. Our results showed that the S211 primer can identify the male, and in doing so, it facilitates buffalo grass breeding work.

Key words: Buffalo grass; RAPD; Sex identification

INTRODUCTION

The factors that determine the sex of individuals of many plant species have been the focus of considerable research over the last century (Durand and Durand, 1991). Several factors, including sex chromosomes (Parker and Clark, 1991; Ye et al., 1991), differences in the expression of sex-related genes (Davies and Schwarz-Sommer, 1994), hormones (Sun et al., 1992), and environmental stimuli have been linked to sex determination in different species.

Buffalo grass belongs to a monospecific genus in the tribe Chlorideae (Poaceae). It is a perennial, sod-forming short grass that is indigenous to the grasslands of central North America, ranging from Montana to northern Mexico (Hitchcock and Chase, 1951). It has been widely used as a turf grass in northern China ever since it was introduced in 1944 to TianShui in Gansu. There are no morphological differences in the early vegetative growth of the three sex classes (viz., male, female, or hermaphrodite) of this plant. This poses a challenge for buffalo grass breeders to identify the sex of a single plant.

Approximately 4% of all angiosperm species are dioecious (Yampolsky and Yampolsky, 1922), which means that male and female organs are found in distinct individuals. The factors that determine the sex of individual members of these species are intriguing (Ćulafić et al., 1987). Although a few dioecious plants have identifiable sex chromosomes (Wilby and Parker, 1986; Vyskot and Hobza, 2004), there is no reported evidence of sex chromosomes in buffalo grass. This may be attributed in part to the fact that karyotype analysis of buffalo grass is complicated by its ability to exist in diploid ($2n = 20$), tetraploid, or hexaploid states (Johnson et al., 1998).

The identification of characteristic isoenzymes has been used for the early determination of sex in certain dioecious species such as *Spinacia oleracea* (Penel and Grepin, 1972), poplar (Poonam et al., 2006), *Hippophae salicifolia* (Poonam et al., 2009), *Carica papaya* (Coppens d'Eeckenbrugge et al., 2007), *Simmondsia chinensis* (Suganuma, 1999), and *Phoenix dactylifera* (Suganuma and Iwasaki, 1983). However, the isoenzyme profile is sensitive to changes in the environment and is also affected by the type and age of the tissue sample analyzed.

Nucleic acid-based molecular makers are more stable than isoenzymes as markers of traits, and their use is less labor intensive than karyotype analysis. The random amplified polymorphic DNA (RAPD) (Shirkot et al., 2002; Dnyaneshwar et al., 2006), amplified fragment length polymorphism (Jamsari et al., 2004; Jamsari et al., 2004; Paolucci et al., 2010), simple sequence repeat (Glawe and De Jong, 2005; Khattak et al., 2006; Khattak et al., 2006), sequence-related amplified polymorphism (Zhou et al., 2011), and sequence-characterized Aamplified Rregion (Yakubov et al., 2005) classes of nucleic acid

markers have all been used to identify the sex of species. Although many studies have been conducted on molecular markers for buffalo grass (Huff et al., 1993; Jiang and Sink, 1997; Budak et al., 2004a,b; Zhang et al., 2007) they do not describe the sex determination aspect. The goal of this study was to use an RAPD marker to identify the sex of buffalo grass plants.

MATERIAL AND METHODS

Plant materials

Of 83 6-year-old plants of known sex, we collected tissue samples from 23 plants (9 male, 10 female, and 4 monoecious individuals), which had a stable sex for more than 3 years. These tissue samples were used for the preparation of genomic DNA for analysis using RAPD markers.

In August 2010, we germinated 188 seeds (Cultivar: Spark, bought from the Clover Co., Beijing), under greenhouse conditions at China Agriculture University. The seedlings were transferred to a farm in Tai Zhou Wu near China Agriculture University for growth under field conditions. These plants were used as a source of DNA to evaluate the utility of the RAPD marker identified from a preliminary screen.

Genomic DNA isolation

Leaf samples were kept in a box containing ice and transported to the laboratory, where genomic DNA was isolated using the DNA Secure Plant Kit (Tiagen Biotech Co., Ltd; Beijing). Plant tissue (100 mg fresh weight) was ground thoroughly under liquid nitrogen. Then, 400 μ L of buffer LP1 and 4 μ L, 10 mg/mL RNase A were added to the powered plant tissue, and the mixture was vortexed for 1 min to mix thoroughly, making sure to disperse all clumps of tissue. The samples were then incubated for 10 min at room temperature. Subsequently, 130 μ L buffer LP2 was added to the lysate and the microcentrifuge tube was again mixed thoroughly by vortexing for 1 min. The contents of the tube were centrifuged for 5 min at 12,000 rpm. The supernatant was transferred to a new tube, to which 1.5 volumes of buffer LP3 were added followed by immediately mixing by vortexing for 15 s. A precipitate was often formed after the addition of the LP3 buffer. All of the mixture from the previous step, including any precipitate that may have formed, was transferred to the spin column CB3, where the contents were centrifuged for 30 s at 12,000 rpm and the flow-through was discarded. After placing the spin column CB3 back in the collection tube, 700 μ L PW solution was added to wash the membrane, and the contents were centrifuged for 30 s at 12,000 rpm. After discarding the flow-through, the spin column CB3 was replaced in the collection tube, and 500 μ L of ethanol was added to the spin column CB3, and the set-up was centrifuged for 30 s at 12,000 rpm. The flow-through was discarded, before the second replacement of the spin column CB3 in the collection tube. We then added 500 μ L of PW solution to the spin column CB3 to wash the membrane and centrifuged for 30 s at 12,000 rpm before discarding the flow-through. The spin column CB3 in the collection tube was replaced again and the contents were centrifuged for 30 s at 12,000 rpm to remove residual wash buffer PW; then, and the collection tube was

discarded. The spin column CB3 was transferred to a clean 1.5 mL microcentrifuge tube, leaving the lid of the spin column open at room temperature for several minutes to dry the membrane completely. Subsequently, 120 μ L TE buffer was pipetted directly onto the CB3 membrane, and the column was incubated for 5 min at room temperature, after which the contents were centrifuged for 2 min at 12,000 rpm to elute the DNA.

Polymerase chain reaction (PCR)

Amplification of the DNA was carried out in a 0.7 mL PCR tube using a 25 μ L reaction volume. Each tube contained 2.5 μ L plant DNA, 12.5 μ L 2X Taq PCR mix (0.1U/ μ L Taq polymerase, 500 μ M each dNTP, 200 mM Tris-HCl, pH 8.3, 100 mM KCl, 3 mM MgCl₂), 0.5 μ L RAPD primer, and 9.5 μ L ddH₂O. The 300 primers used were named s1, s2, s3, s4...s300.

A thermocycler (Single Block SystemEasy Cycler; Ericomp Inc. San Diego, California) was set to run through 45 cycles, each comprising 1 min at 94°C (denaturation), 45s at 37°C (annealing), and 2 min at 72°C (extension). This was followed by a final extension step increased to 10 min; thereafter, the reaction products were held at 4°C. The amplification products were analyzed by electrophoresis on a 1.7% agarose gel containing 0.5X TBE. The well at the end of every set of samples corresponding to one of the sexes examined was reserved for loading a size marker (D2000;) (Tiangen Biotech Co, LTD; Beijing). Gels were run at 160 V until the dye reached the end of the gel (approximately 2 h). After staining with ethidium bromide, the DNA bands were visualized using a transilluminator, and the gel was photographed. All RAPD reactions were repeated at least twice.

Cloning and sequencing

After separation of the PCR products on the agarose gel, the gel parts corresponding to bands of interest were collected and the DNAs extracted using the TIAN Gel Midi Purification Kit (Tiangen Biotech Co.). For column equilibration, 500 μ L buffer BL was added to the spin column CA2, and the contents were centrifuged for 1 min at 12,000 rpm using a table-top microcentrifuge, and the flow-through was discarded. The spin column CA2 was next placed into a collection tube, and excise the single DNA fragment was excised from the agarose gel with a clean, sharp scalpel, after which 3 volumes of buffer PN were added to the agarose gel sample. The mixture was incubated in a 50°C water bath for about 10 min, while periodically pipetting the solution up and down until the agarose gel had dissolved completely. When the gel was completely dissolved and the solution had returned to room temperature, the whole solution was transferred to the spin column CA2, allowed to stand for 2 min at room temperature it was then centrifuged for 60 s at 12,000 rpm using a table-top microcentrifuge. After discarding the flow-through, the spin column CA2 was replaced in the collection tube, and the column was washed by adding 600 μ L buffer PW and centrifuging for 60 s at 12,000 rpm. Again, after discarding the flow-through, the spin column CA2 was placed into a clean 1.5-mL microcentrifuge tube. To elute the DNA, we added 40 μ L buffer EB was added to the center of each spin column CA2, and the solution was allowed to stand for 2 min; the contents of the tube were then centrifuged for 2 min at 12,000 rpm.

The fragment was subcloned into the PCR2.1 vector (Invitrogen) and then trans-

formed into Trans1-T1 Phage Resistant *Escherichia coli* cells (Beijing TransGen Biotech Co., Ltd). Positive clones were sequenced using the M13F and M13R primers. Sequences were aligned using the DNAMAN software.

RESULTS

Using 300 primers to amplify genomic DNA from 23 buffalo grass plants, we identified approximately 1200 discrete DNA fragments following agarose electrophoresis. Notably, the amplification of genomic DNA of buffalo grass using the S211 primer produced a 833bp fragment that was amplified from genomic DNA isolated from nine male plants, but not from DNA isolated from four monoecious plants or 10 female plants (Figure 1). The specific fragment was cloned, sequenced, and submitted to the GenBank database (accession No. JN982469).

Figure 1. RAPD primer S211 amplifies a 833-bp fragment from genomic DNA isolated from male buffalo grass plants, but not from female or monoecious buffalo grass plants.

A similarity search using the NCBI BLAST program revealed no gene with a similarity as high as 50%. Therefore, this gene is likely to be a novel gene.

Using InterProScan to predict the gene function, it was found that the gene has three open reading frames: the first one is a part of the SignalP-NN (euk) signal peptide; the second is a part of a transmembrane region; and the third is a part of the retrotransposon Gag protein.

We isolated DNA from 188 additional plants to confirm the utility of the S211 primer to identify male plants. Of these, 92 were male plants, 29 were monoecious, and 63 were female plants, as identified from their flowers in the first growth season.

During the course of the verification test, the S211 primer correctly identified the sex of 79 male plants in all 92 male plants, although DNA isolated from female plants was never amplified. A 883 bp fragment amplified from DNA isolated from almost all male plants was also amplified from two monoecious plants (Figure 2).

Figure 2. Representative results from amplification of DNA isolated from 188 buffalo grass plants using the S211 primer. Although never amplified from DNA isolated from female plants, a 883-bp fragment amplified from DNA isolated from almost all male plants was also amplified from DNA isolated from two monoecious plants.

DISCUSSION

Buffalo grass plants are now generally accepted to be male, female, or hermaphrodite. Nonetheless, there have been many different proposals regarding the reproductive biology of buffalo grass. Whereas certain botanists have argued that the species is exclusively monoecious (Gernert, 1937; Durham, 1942), others consider the plant to be largely unisexual and occasionally monoecious (Savage, 1934; Wenger, 1943). To the best of our knowledge, the most effective way to identify the sex of any single buffalo grass plant is to classify it as being either female, mostly female, monoecious, mostly male, or male (Quinn and Engel, 1986; Huff and Wu, 1992). There is also evidence that buffalo grass plants may change their sex across this continuum over time. For instance, Davies and Schwarz-Sommer (1994) reported that certain plants, which he called “inconstant male” and “inconstant female”, can change their sex. Quinn and Engel (1986) reported that of the 74 female plants they examined, four needed to be reclassified as “mostly female” within a year of the initial analysis; furthermore, two of these four plants, classified as female and two as “mostly female” within the next year. Over

the course of a year, five buffalo grass plants originally classified as monoecious needed to be reclassified as four female plants and one monoecious plant, and three “mostly male” plants needed to be reclassified as two female plants and one “mostly male” plant. Moreover, 56 male plants were transformed to three “mostly male” plants and 53 male plants. Given that there is no way to unambiguously identify the sex of any buffalo grass plant, the 12 male plants that could not be marked by S211 may well change their sex during the years; hence, we suspect that the 85.8% success rate estimated in our study for identifying male plants in our confirmatory test is good. In this study, samples were classified as being female, male, or monoecious. Given that the “inconstant female” and “inconstant male” samples were considered as samples from plants classified as monoecious, it is not altogether surprising that the marker associated with male, but not female, individuals was detected in two monoecious plants.

Why does this sex gene have little similarity with the other genes? We believe that many of the genes which marked with sex are just located on the sex chromosome, but they are not invoked with sex determination. The result of function prediction is based only on the BLAST comparison, which cannot predict the real function.

Thus, the molecular basis of sex determination in buffalo grass remains to be elucidated. There may be several genes linked to sex, as in maize (Huff and Wu, 1992; Banks, 2008; Banks, 2008; Acosta et al., 2009; Freedberg et al., 2011; Yasukochi et al., 2011), or other mechanisms may be involved. Future research in this regard might, for instance, focus on trying to identify a sex chromosome(s) or factors that influence the differentiation of flower buds.

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REFERENCES

- Banks JA (2008). MicroRNA, sex determination and floral meristem determinacy in maize. *Genome Biol.* 9: 204.
- Budak H, Shearman RC, Parmaksiz I, Gaussoin RE, et al. (2004a). Molecular characterization of Buffalograss germplasm using sequence-related amplified polymorphism markers. *Theor. Appl. Genet.* 108: 328-334.
- Budak H, Shearman RC, Parmaksiz I and Dweikat I (2004b). Comparative analysis of seeded and vegetative biotype buffalograsses based on phylogenetic relationship using ISSRs, SSRs, RAPDs, and SRAPs. *Theor. Appl. Genet.* 109: 280-288.
- Coppens d'Eeckenbrugge G, Restrepo MT, Jimenez D and Mora E (2007). Morphological and isozyme characterization of common papaya in Costa Rica. *Acta Hort.* 740: 109-120.
- Čulafić L, Samofalova A and Nešković M (1987). *In vitro* organogenesis in two dioecious species, *Rumex acetosella* L. and *R. acetosa* L. (Polygonaceae). *Plant Cell, Tissue Organ Cult.* 11: 125-131.
- Davies B and Schwarz-Sommer Z (1994). Control of floral organ identity by homeotic MADS-box transcription factors. *Res. Probl. Cell Differ.* 20: 235-258.
- Dnyaneshwar W, Preeti C, Kalpana J and Bhushan P (2006). Development and application of RAPD-SCAR marker for identification of *Phyllanthus emblica* LINN. *Biol. Pharm. Bull.* 29: 2313-2316.
- Durand B and Durand R (1991). Sex determination and reproductive organ differentiation in *Mercurialis*. *Plant Sci.* 80: 49-65.
- Durham SB (1942). Buffalograss. Leaflet of the Oklahoma Cooperative Extension Service. OP-23.
- Freedberg S, Lee C and Pappas M (2011). Agricultural practices alter sex ratios in a reptile with environmental sex determination. *Biol. Conserv.* 144: 1159-1166.
- Gernert WB (1937). Variation in buffalo grass. *Am. Soc. Agron.* 29: 242-246.

- Glawe GA and De Jong TJ (2005). Environmental conditions affect sex expression in monoecious, but not in male and female plants of *Urtica dioica*. *Sex. Plant Rep.* 17: 253-260.
- Hitchcock AS and Chase A (1951). Manual of the grasses of The United States: Hitchcock; rev. by Agnes Chase: United States Government Printing Office. *Hippophae salicifolia* using isozymes as sex markers. *Ind. J. For.* 32: 231-237.
- Huff DR and Wu L (1992). Distribution and inheritance of inconstant sex forms in natural populations of dioecious buffalograss (*Buchloe dactyloides*). *Am. J. Bot.* 79: 207-215.
- Huff DR, Peakall R and Smouse PE (1993). RAPD variation within and among natural populations of outcrossing buffalograss [*Buchloe dactyloides* (Nutt.) Engelm.]. *Theor. Appl. Genet.* 86: 927-934.
- Jamsari A, Nitz I, Reamon-Buttner SM and Jung C (2004). BAC-derived diagnostic markers for sex determination in asparagus. *Theor. Appl. Genet.* 108: 1140-1146.
- Jiang C and Sink KC (1997). RAPD and SCAR markers linked to the sex expression locus *M* in asparagus. *Euphytica* 94: 329-333.
- Johnson PG, Riordan TP and Arumuganathan K (1998). Ploidy level determinations in buffalograss clones and populations. *Crop Sci.* 38: 478-482.
- Khattak JZK, Torp AM and Andersen SB (2006). A genetic linkage map of *Spinacia oleracea* and localization of a sex determination locus. *Euphytica* 148: 311-318.
- Paolucci I, Gaudet M, Jorge V, Beritognolo I, et al. (2010). Genetic linkage maps of *Populus alba* L. and comparative mapping analysis of sex determination across *Populus* species. *Tree Genet. Genomes* 6: 863-875.
- Parker JS and Clark MS (1991). Dosage sex-chromosome systems in plants. *Plant Sci.* 80: 79-92.
- Penel C and Greppin H (1972). Development of auxin oxidase and peroxidase activity during photoperiodic induction and sex determination in spinach. *Plant Cell Physiol.* 13: 151-156.
- Poonam S, Vasudha, Sharma DR and Rachana (2006). Use of isozyme polymorphism for sex identification in the dioecious poplars. *J. Hill Res.* 19: 34-36.
- Poonam S, Monika B and Gaurav Z (2009). Identification of gender in dioecious *Hippophae salicifolia* using isozymes as sex markers. *Ind. J. For.* 32: 231-237.
- Quinn JA and Engel JL (1986). Life-history strategies and sex ratios for a cultivar and a wild population of *Buchloe dactyloides* (Gramineae). *Am. J. Bot.* 73: 874-881.
- Savage DA (1934). Methods of Reestablishing Buffalograss on Cultivated Land in the Great Plains. 1st edn. U.S. Department of Agriculture, Washington, D.C.
- Shirkot P, Sharma DR and Mohapatra T (2002). Molecular identification of sex in *Actinidia deliciosa* var. *deliciosa* by RAPD markers. *Sci. Hortic.* 94: 33-39.
- Suganuma H (1999). Sex identification of dioecious jojoba plants by isozyme method. *Jpn. J. Trop. Agric.* 43: 103-106.
- Suganuma H and Iwasaki F (1983). Sex identification of dioecious plants by the isozyme method. Date (*Phoenix dactylifera* L.). *Jpn. J. Trop. Agric.* 27: 75-78.
- Sun T, Goodman HM and Ausubel FM (1992). Cloning the *Arabidopsis* GA1 locus by genomic subtraction. *Plant Cell* 4: 119-128.
- Vyskot B and Hobza R (2004). Gender in plants: sex chromosomes are emerging from the fog. *Trends Genet.* 20: 432-438.
- Wenger LE (1943). Buffalograss. Manhattan, Kan.: Agricultural Experiment Station, Kansas State College of Agriculture and Applied Science.
- Wilby AS and Parker JS (1986). Continuous variation in Y-chromosome structure of *Rumex acetosa*. *Heredity* 57: 247-254.
- Yakubov B, Barazani O and Golan-Goldhirsh A (2005). Combination of SCAR primers and Touchdown-PCR for sex identification in *Pistacia vera* L. *Sci. Hortic.* 103: 473-478.
- Yampolsky C and Yampolsky H (1922). Distribution of Sex Forms in the Phanerogamic Flora. 1st edn. Gebruder Borntraeger Press, Leipzig.
- Yasukochi Y, Miura N, Nakano R, Sahara K, et al. (2011). Sex-linked pheromone receptor genes of the European corn borer, *Ostrinia nubilalis*, are in tandem arrays. *PLoS One* 6: 18843.
- Ye D, Oliveira M, Veuskens J, Wu Y, et al. (1991). Sex determination in the dioecious *Melandrium*. The X/Y chromosome system allows complementary cloning strategies. *Plant Sci.* 80: 93-106.
- Zhang XB, Su DR and Luyi M (2007). Analysis of genetic diversity in buffalograss determined by random simplified polymorphic. *HortScience* 42: 474-477.
- Zhou YJ, Wang XG and Zhang XQ (2011). Development and application of a SRAP marker for the identification of sex in *Buchloe dactyloides*. *Euphytica* 181: 261-266.