Theor Appl Genet (2010) 121:919–929
DOI 10.1007/s00122-010-1360-3

ORIGINAL PAPER

Apomixis and ploidy barrier suppress pollen-mediated gene flow in field grown transgenic turf and forage grass (*Paspalum notatum* Flüggé)

Sukhpreet Sandhu · Ann R. Blount · Kenneth H. Quesenberry · Fredy Altpeter

Received: 28 December 2009 / Accepted: 12 May 2010 / Published online: 30 May 2010

Abstract Bahiagrass (*Paspalum notatum* Flüggé) is the predominant forage grass in the southeastern US. The commercially important bahiagrass cultivar 'Argentine' is preferred for genetic transformation over sexual diploid cytotypes, since it produces uniform seed progeny through apomixis. Pseudogamous apomictic seed production in Argentine bahiagrass may contribute to transgene confinement. It is characterized by embryo development which is independent of fertilization of the egg cell, but requires fertilization with compatible pollen to produce the endosperm. Pollen-mediated gene transfer from transgenic, glufosinate-resistant apomictic bahiagrass as pollen donor at close proximity (0.5–3.5 m) with non-transgenic sexual or apomictic bahiagrass cultivars as pollen receptors was evaluated under field conditions. Hybridization frequency was evaluated by glufosinate herbicide resistance in >23,300 seedlings derived from open-pollinated (OP) pollen receptor plants. Average gene transfer between transgenic apomictic, tetraploid and sexual diploid bahiagrass was 0.03%. Herbicide-resistant hybrids confirmed by immuno-chromatographic detection of the PAT protein displayed a single copy *bar* gene identical to the pollen parent. Hybrids resulting from diploid pollen receptors were confirmed as triploids or aneu-triploids with significantly reduced vigor and seed set as compared to the parents. Transmission of transgenes to sexual bahiagrass is severely restricted by the ploidy difference between tetraploid apomicts and diploid sexual bahiagrass. Average gene transfer between transgenic apomictic tetraploid and non-transgenic, apomictic tetraploid bahiagrass was 0.17%, confirming a very low frequency of amphimixis in apomictic bahiagrass cultivars. While not providing complete transgene containment, gene transfer between transgenic apomictic and non-transgenic bahiagrass occurs at a much lower frequency than reported for other cross-pollinating or facultative apomictic grasses.

Communicated by P. Heslop-Harrison.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-010-1360-3) contains supplementary material, which is available to authorized users.

S. Sandhu · K. H. Quesenberry · F. Altpeter (✉)
Plant Molecular and Cellular Biology Program,
Agronomy Department, Genetics Institute,
University of Florida, IFAS, Gainesville, FL, USA
e-mail: altpeter@ufl.edu

A. R. Blount
North Florida Research and Education Center,
University of Florida, IFAS, Marianna, FL, USA

Present Address:
S. Sandhu
Center for Applied Genetic Technologies,
Institute of Plant Breeding Genetics and Genomics,
University of Georgia, Athens, GA, USA

Introduction

Unintended intra- or inter-specific transfer of specific transgenes could potentially result in environmental, agronomic or health problems (Jorgensen et al. 2009). The magnitude of transgene dispersal under field conditions will depend on the genetically modified (GM) crop, the agricultural practice and the environment of the release site (Chapman and Burke 2006). Some of the common factors which can influence the frequency of gene transfer include: (1) spatial overlap or proximal geographic location between pollen receptors and pollen donor plants (Messeguer et al. 2004; Cunliffe et al. 2004); (2) pollen longevity

and dispersal (Wang et al. 2004, 2006; Rong et al. 2007); (3) asynchronous flowering of transgenic plants and their non-transgenic counterparts either naturally or induced by frequent mowing of turf during the time of flowering might prevent successful hybridization (Messeguer et al. 2006; Hoyle et al. 2007). In the present study, we investigated the transmission of transgenes from an apomictic transgenic pollen donor.

Apomixis is a common phenomenon found in 300 species across 40 families, where embryo development precludes pollen fertilization of the egg cell. Apomictic seed production is a useful mechanism for fixing hybrid vigor and maintaining purity of superior genotypes (Hanna and Burton 1986). However, lack of genetic recombination in apomictic species is linked to reduced genetic variability and has been a stumbling block in the genetic improvement of apomictic cultivars through traditional breeding techniques (Bashaw and Funk 1987; Hanna 1995). Transgenic approaches for crop improvement have been reported from several apomictic species including bahiagrass (*Paspalum notatum* Flüggé; Smith et al. 2002; Altpeter and James 2005; Agharkar et al. 2007; James et al. 2008; Sandhu et al. 2007; Zhang et al. 2007), Kentucky bluegrass (*Poa pratensis* L.; Ha et al. 2001; Gao et al. 2006), *Dichanthium annulatum* Forssk (Dalton et al. 2003; Kumar et al. 2005), buffelgrass (*Cenchrus ciliaris* L.; Batra and Kumar 2003) and blue grama grass [*Bouteloua gracilis*; (HBK) Lag. Ex Steud. Aguado-Santacruz et al. 2002]. There is limited information on pollen-mediated gene flow from transgenic apomictic species. Inter-specific gene transfer frequencies from the facultative apomictic Kentucky bluegrass (*Poa pratensis* L.) to 25 *Poa* spp. have been reported to average 0.53% in close proximity (0 m) of pollen donor and receptor, while intra-specific gene transfer reached a maximum hybrid frequency of 16% (Johnson et al. 2006).

Bahiagrass is extensively used as pasture, and as utility turf in Florida and the southeastern United States (Chambliss and Adjei 2006; Gates et al. 2004). Chromosome numbers of $2n = 20$ or 40 are typically reported for bahiagrass (Burton 1946; Saura 1948; Gould 1966). The diploid cytotype (*P. notatum* var. *saurae*), known as 'Pensacola' type bahiagrass, is most widely grown in the USA and is sexual and cross-pollinating due to self-incompatibility (Quarin et al. 2001). The most dominant bahiagrass cytotype in South America near its center of genetic diversity is tetraploid ($2n = 40$) (Burson and Watson 1995; Pozzobon and Valls 1997). Autoploidization is considered as the origin of tetraploid races based on quadrivalent chromosome associations (Forbes and Burton 1961; Quarin et al. 1984; Stein et al. 2004). The tetraploid races are considered obligate apomicts (Burton 1948). However, this statement is based on embryo-sac analysis and should be confirmed by seed progeny analysis. Argentine, a commercially important tetraploid apomictic bahiagrass cultivar is preferred for genetic transformation since its asexual seed production prevents transgene segregation (Sandhu and Altpeter 2008). Apomictic bahiagrass cultivars are known to produce a high percentage of viable pollen, which is required for the formation of the endosperm by fusion with the central cells, termed pseudogamy (Burton and Forbes 1960; Quarin 1999). Therefore, pollen from transgenic apomictic bahiagrass could contribute to gene flow to non-transgenic apomictic or sexual bahiagrass. Greenhouse evaluation of short distance gene transfer from transgenic to non-transgenic bahiagrass identified a very low hybridization frequency of 0.16% (Sandhu et al. 2009). A field study where non-transgenic, apomictic tetraploid or sexual diploid bahiagrass pollen receptors surround the transgenic apomictic tetraploid pollen donor at short distances (0.5–3.5 m) will allow the detection of infrequent gene transfer. Gene transfer between transgenic and non-transgenic apomictic tetraploid bahiagrass will evaluate the degree of sexuality/apomixis in tetraploid bahiagrass cultivars. Gene transfer between transgenic tetraploid and non-transgenic sexual diploid cultivars will evaluate if the ploidy barrier prevents gene transfer from the transgenic pollen source effectively under field conditions. Although intra-specific hybrids involving *Paspalum notatum* and resulting in triploid cytotypes are known to occur naturally (Burson and Watson 1995), quantitative assessments of relative hybridization frequencies under field conditions are lacking. The impact of a ploidy barrier or apomixis on pollen-mediated gene flow is not fully understood. Our objective was to quantify inter-specific pollen-mediated gene flow from apomictic tetraploid bahiagrass to tetraploid or diploid bahiagrass by testing hybridization potential under field conditions using herbicide resistance as a selectable marker.

Experimental procedures

Field layout and seed head collection

The field study was designed to evaluate the hybridization frequency between different bahiagrass cytotypes at a short distance (0.5–3.5 m). The field study was conducted for two consecutive years (2005 and 2006) at the UF-IFAS research center near Marianna, FL. Herbicide-resistant transgenic apomictic bahiagrass line (B9) was used as pollen donor (Sandhu et al. 2009). Pollen viability of the field planted B9 plants was tested by aceto-orcein staining as described in Sandhu et al. 2009. Transgenic apomictic pollen donor plants (24 whole plants/plot) were planted at the center, while the non-transgenic pollen receptors were planted in a circle of 2 m diameter surrounding the pollen

donors (Figs. 1, 2). Each circular plot was demarcated into four quadrants representing wind direction (SE, SW, NE and NW). Two plots represented replications of the experiment, which had 64 diploid cv. 'Pensacola' plants at 1 m radius from the inner circle of pollen donor plants. In the other two plots, 64 wild-type tetraploid bahiagrass cultivars were planted as pollen receptors. The tetraploid cultivars including 'Argentine', 'Wilmington', 'Paraguay 22' and an experimental accession 'Tifton 7' were selected based on their abundance and/or availability in the USA. All plants were clonally propagated in greenhouses and equal numbers of each tetraploid cultivar were transplanted to the four quadrants of the field representing wind direction in May 2005. The field study was conducted in compliance with the USDA-APHIS guidelines (notification no. 05-076-11n and permit no. 05-364-01r). Permit no. 05-364-01r was issued following an environmental assessment and public commenting period. To prevent seed dispersal by birds or animals, cage-like structures were built on a wooden scaffold with mosquito net. An isolation zone of 30 ft around the circular plots was maintained plant free by monthly application of glyphosate. A 300 ft area surrounding the circular plot was kept mowed weekly to prevent establishment of hybrids outside the plot area.

Weather data observations recorded every 15 min were obtained from the Florida Automated Weather Network (FAWN). Parameters included temperature, relative humidity and wind speed and wind direction.

All plants were monitored every 3–4 days at the start of flowering (end of June to beginning of July) to identify the peak period for synchronous flowering of pollen donors and receptors. During 3 weeks of the peak flowering period emerging seed heads were tagged with their emergence date every 2 days and bagged in glassine pollination bags after 7–10 days of pollination to prevent seed shattering (Fig. 2). Mature seed heads were harvested 28–35 days after pollination (end of August to beginning of September). Seed heads from the transgenic pollen parent were also bagged and harvested separately as positive control. Seed heads were air-dried, threshed and cleaned manually.

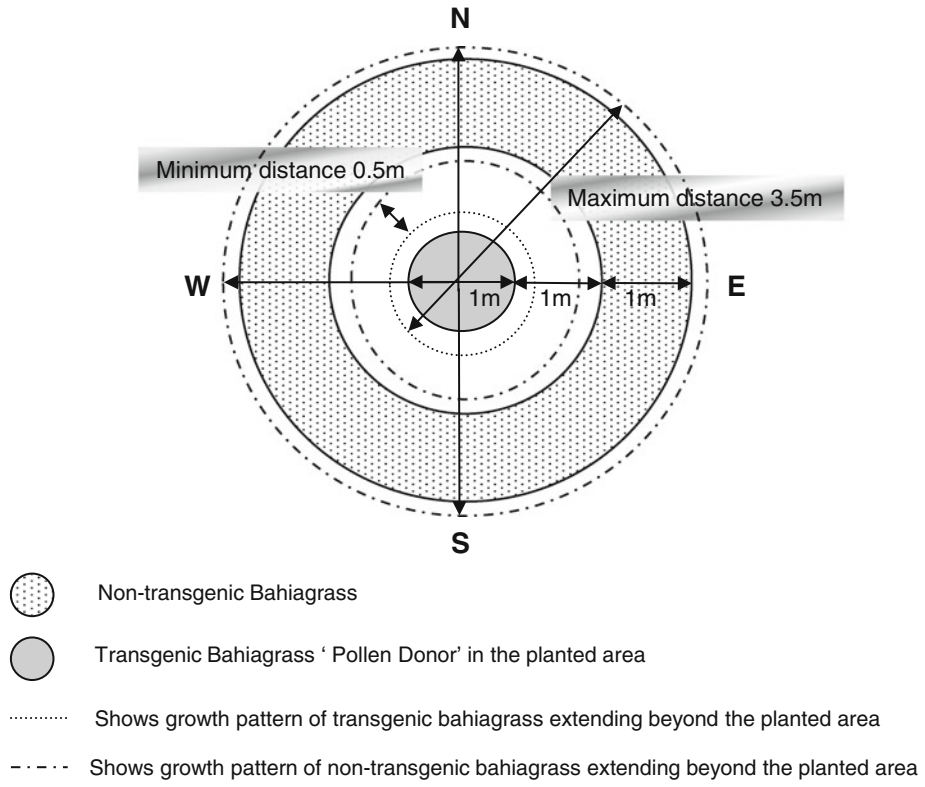

Fig. 1 Experimental plan for the gene flow field study located at Marianna FL (GPS coordinates 30°52′ 36.14″ and 85°11′ 22.83″). A circular field plot design consisted of tetraploid transgenic plants (pollen source) in the center surrounded by wild-type non-transgenic bahiagrass plants (pollen receptors). Seed harvested from each of the four quadrants (NE, NW, SE and SW) was collected separately. The minimum distance (0.5 m) describes pollen that travels from inflorescences of transgenic plants to the closest inflorescences of the non-transgenic plants, considering that the mature inflorescences extend beyond the originally planted area. The maximum distance (3.5 m) describes pollen that travels from inflorescences of transgenic plants to the most distant inflorescences of the non-transgenic plants, considering that the mature inflorescences extend beyond the originally planted area

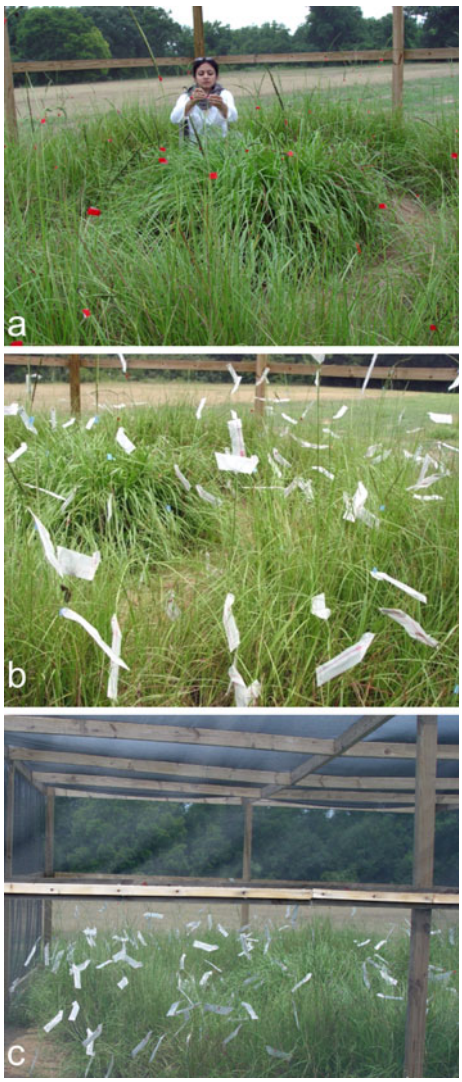

Fig. 2 Transgenic and non-transgenic bahiagrass at the time of flowering in the field. **a** Bahiagrass seed heads tagged at anthesis. **b** Bahiagrass seed heads bagged in glassine pollination bags 7–10 days after pollination. **c** An outside view of the cages built over the circular field plots to prevent seed dispersal through birds and animals

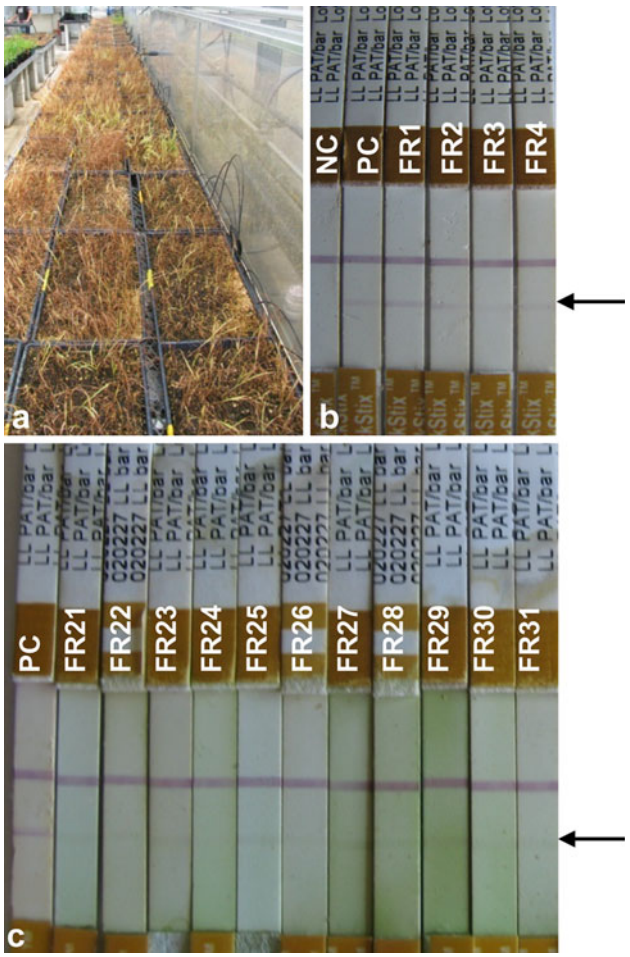

Fig. 3 Hybrid detection and confirmation. **a** OP seed progeny screened for the hybrids by herbicide application. Seedlings were grown in the greenhouse and treated with 0.14% Ignite® herbicide, 6 weeks after germination. The herbicide-resistant hybrids were scored 3 weeks after herbicide application. **b** Immunochromatographic determination of bar/PAT expression using Quick Stix™ LibertyLink®/bar (Envirologix, Portland, ME) with crude extracts from herbicide-resistant hybrids derived from diploid pollen receptors (FR1-4) wild-type bahiagrass (NC) or transgenic bahiagrass B9 (PC). Protein extracts from the wild type only show the *top band,* while *bar*/PAT is detected by the bottom PAT-specific band (indicated by the *arrow*). **c** Immunochromatographic determination of *bar*/PAT expression using Quick Stix™ LibertyLink®/bar (Envirologix, Portland, ME) with crude extracts from herbicide-resistant hybrids derived from tetraploid pollen receptors (FR21-31) or transgenic bahiagrass B9 (PC)

Seedling evaluation

Seed progeny from wild-type diploids and tetraploids were screened for herbicide resistance to detect gene flow. A weighed quantity (2.0 g) of seeds was treated with concentrated sulfuric acid and washed four to five times with deionized water. Seeds were germinated in flats filled with Fafard 2 potting mix (Fafard Inc., Apopka, FL) and maintained at 28 ± 2°C under natural photoperiod. Wild-type bahiagrass seeds and herbicide-resistant transgenic bahiagrass seeds from line B9 were used as NC and PC, respectively. Seedlings at the 6-week stage were sprayed with 0.14% glufosinate ammonium (Ignite®, ArgEvo, Wilmington, DE). Seedlings surviving herbicide application were transplanted to pots and maintained in the greenhouses for further characterization (Fig. 3a). Herbicide application was optimized at the lowest concentration (0.14% glufosinate ammonium) which produced 80–100% necrosis on the wild-type non-transgenic bahiagrass, and did not produce any injury symptoms on the herbicide-resistant transgenic seed progeny. Seedlings were counted prior to herbicide application.

Hybrid characterization

Immunochromatography assay for transgene expression

All herbicide-resistant plants were confirmed for *bar* gene expression using a lateral immunochromatography assay as described in Sandhu et al. (2007).

Southern blot analysis for transgene integration

Genomic DNA was extracted using the modified CTAB protocol for DNA extraction (Murray and Thompson 1980). Genomic DNA digested with *Eco*RI was electrophoresed overnight and blotted to nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) according to standard procedures (Sambrook and Russell 2001). Hybridization using 25 ng [^{32}P] dCTP radio-labeled *bar* coding sequence as probe was performed as described in Sandhu et al. (2007).

RFLP analysis for mode of reproduction

The procedure for restriction fragment length polymorphism (RFLP) was similar to Southern blotting with minor modifications in the washing steps. Genomic DNA was digested with restriction enzyme *Hin*dIII. Hybridization with rice probe C1069 allowed the determination of apomictic or sexual nature of hybrids (Martínez et al. 2003). The C1069 probe was amplified from rice genomic DNA. Sequence information available at the NCBI database was used to design sequence-specific primers (forward primer: 5′ ACAAGTCACACGGGATCACA 3′; reverse primer: 5′ TCCTCTGCACGGATTCTCTT 3′). The amplified PCR product was sequenced by MWG (MWG-Biotech Inc., High Point NC) using the specific primers. Hybridized membrane was washed as follows: (1) quick wash using 2× SSC; (2) 20 min low stringency wash using 2× SSC, 0.5% SDS at 65°C; and (3) 20-min high stringency wash using 0.2× SSC, 0.1% SDS at 65°C. The membrane was exposed to autoradiographic film (Kodak Biomax MS autoradiography) for 48 h at −80°C.

Ploidy determination using flow cytometry and chromosome counting

Ploidy of the hybrid progeny obtained from OP sexual diploid plants was determined using flow cytometry and confirmed by chromosome counting. Fresh meristematic apical region (5 mm) of the leaf was chopped in 500 µl nuclei extraction buffer (CyStain® PI Absolute P; North America Inc., Mt. Laurel, NJ). The material was filtered using a 50-µm filter followed by the addition of a drop of the chicken erythrocytes nuclei (CEN) as internal standard (Biosure Inc., Grass Valley, CA). To the filtrate, 2 ml propidium iodide (PI) stain was added. Samples were incubated for 1 h in dark at 4°C before reading on a CyFlow® space Flow Cytometry System (Partec North America Inc., Mt. Laurel, NJ). The analyzer was arbitrarily calibrated using known tetraploid bahiagrass and a known diploid bahiagrass. The DNA index was obtained by dividing the sample fluorescence with standard DNA (CEN) fluorescence. Three independent nuclei extractions were used for each sample and histograms showing >10% CV were ignored.

Root-tip preparations were made by 4 h pretreatment with 8-hydroxyquinoline followed by overnight treatment with the fixative (95% ethanol: acetic acid, 3:1 v/v). Fixed root material was stored in 70% ethanol. The root tip was chopped (2 mm) in 45% acetic acid and stained with aceto-orcein for 30 min. Mitotic chromosomes were observed under 500× or 1,000× magnification.

Evaluation of plant phenotypes and growth characteristics under hydroponics growth conditions

Pre-weighed single tillers from two hybrid plants (FR1 and FR3), tetraploid wild-type (4×-wt) and diploid wild-type bahiagrass (2×-wt) were transferred to hydroponics growth solution of 1.2% boost and 0.3% grow (Technaflora Plant Products, Port Coquitlam, BC, Canada), pH 6.0. The hydroponic tanks were provided with pressurized air through diffusers to maintain 80% oxygen saturation. The experiment was conducted in an air-conditioned greenhouse under natural photoperiod during June and July 2008 and 28 ± 2°C day and 20 ± 2°C night temperature with four replications in a completely randomized block design. Tiller biomass was recorded 4 weeks after culture initiation from single tillers. At this stage, plantlets were transferred to soil in 6″ pots. Seed head production was recorded at the end of July 2008. Seeds showing normal structure were categorized as viable when compared with undeveloped/empty seeds (i.e. no embryo or endosperm) and viability was confirmed by germination assays as described under seedling evaluation. Data were analyzed according to the randomization plan using the analysis of variance of SAS version 9.1 (SAS Institute). Means were compared by *t* test (LSD, $P < 0.05$).

Results

A field study where non-transgenic, apomictic tetraploid or sexual diploid bahiagrass pollen receptors surrounded the transgenic glufosinate resistant, apomictic tetraploid pollen donor at short distance (0.5–3.5 m; Figs. 1 and 2) allowed the detection of infrequent gene transfer. OP seeds

harvested from the pollen receptor plants were germinated in the greenhouse (Fig. 3a) and sprayed with the herbicide to screen for herbicide-resistant seedlings, which were further evaluated.

Anthesis

Flowering initiation time differed from year 1 to year 2. In 2005 (the establishment year), flowering began late in August and continued till October. In 2006, flowering started by the end of June and peaked between the second and third week of July (more typical of established bahiagrass plants). Plants flowered profusely for 2–3 weeks followed by a drastic reduction in seed head production. For both years, diploid bahiagrass cytotypes started flowering a week to 10 days earlier than the tetraploid cytotypes. This effect was prominent in 2005 when the anthesis of tetraploids started after the peak flowering period of diploids. Both diploids and tetraploids flowered synchronously for 3–4 weeks creating the potential for gene flow. During the peak flowering period, tetraploids produced more seed heads when compared with the diploid cytotypes. Pollen staining showed 60–80% viable pollen production by transgenic B9 plants in the field (data not shown). OP seeds from non-transgenic plants, which were produced during highest seed head production by transgenic B9 plants, were screened for the detection of gene transfer events.

Weather

Weather parameters including wind direction, wind speed, temperature and relative humidity were analyzed for the time period with maximum synchronous flowering of transgenic and non-transgenic bahiagrass plants. Anthesis in bahiagrass occurs during early morning hours, between 6:00 and 8:00 a.m. (Burton 1942). The observations were averaged over time period 4:00–9:00 a.m. Wind speed averaged 3.2 kph in 2005 and 4.8 kph in 2006. In 2005, wind speed was 1.6 kph for 14% of the time, 3.2 kph for 57.5% of the time and 4.8 kph for 28.5% of the time. In 2006, wind speed was 3.2 kph for 28.5% of the time, 4.8 kph for 42.8% of the time and 6.4 kph for 28.5% of the time. For most days, wind originated from the south (S, SW or SSW) in both 2005 and 2006, although a few exceptions when ENE winds were recorded in 2005 (Table S1). In 2006, the prevalent morning winds were ESE, E, SE (frequency 33, 25, 24 respectively). Also WNW winds (frequency 20) with a greater maximum wind speed of 30.5 kph were dominant in the 2006 flowering period. Relative humidity can also affect pollen dispersal (Burton 1942). Average relative humidity during morning hours (4:00–11:00 a.m.) was higher in 2005 (82.3% average over 3 time points) than in 2006 (71.9% average over 3 time points; Table S2).Higher temperatures were recorded during 2006 in comparison with 2005 (average over 3 time points 27.3°C in 2005 and 28.6°C in 2006; Table S3). Mean minimum temperatures recorded over the given time points showed a greater temperature shift in 2006 producing the highest minimum temperature of 30.5°C and lowest minimum of 18.8°C.

Hybridization frequency between transgenic apomictic and non-transgenic bahiagrass

The frequency of hybridization between transgenic apomictic tetraploid and non-transgenic sexual diploid bahiagrass was 0.00–0.05% at 0.5–3.5 m distance. Out of 7,102 seedlings from OP seeds produced in 2005, no herbicide-resistant hybrid plant was detected (0.00%). Five herbicide-resistant hybrid plants were identified from 10,040 seedlings from OP seed produced in 2006 (0.05%). Two out of five herbicide-resistant hybrids originated in the SE, one in the SW and two in the NW direction (Table 1). Gene transfer from transgenic apomictic tetraploid bahiagrass to non-transgenic tetraploid cultivars was 0.17% (11 herbicide-resistant hybrids out of 6,203 seedlings). Two herbicide-resistant putative hybrids were produced in the SE and the remaining nine in the NW direction (Table 1). Out of the 11 herbicide-resistant hybrids, four originated from Argentine, one from Wilmington, five from Paraguay 22 and one from Tifton 7 OP pollen receptor plants.

Hybrid characterization

All putative hybrid plants originating from diploid (Fig. 3b) or tetraploid pollen receptors (Fig. 3c) showed a characteristic band in the immunochromatography lateral membrane flow assay, confirming that the herbicide resistance is due to the presence of the *bar*/PAT.

Southern blot analysis showed a single copy *bar* gene in all three analyzed hybrid plants obtained from OP diploids (Fig. 4a). RFLP marker linked to apomixis (C1069 locus) was detected in all three hybrids obtained from OP diploids (Fig. 4b). Ploidy of hybrids obtained from OP sexual diploid plants was determined by flow cytometry and karyotypic observations. DNA index for known tetraploid and diploid bahiagrass was 0.42 and 0.21, respectively. Test samples FR1 and FR3 showed DNA index of 0.27 and 0.30, respectively. Root-tip chromosome counting showed $2n = 28$ in FR1 and $2n = 30$ in FR3 (Fig. 5). Following greenhouse propagation, the two most vigorous hybrids

Table 1 Seed screening of open-pollinated diploid or tetraploid progeny obtained from four quadrants relative to direction

Diploids	Overall	SE		SW		NE		NWds	
		Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
2005									
Seedlings tested	7,100	576	1,700	1,354	786	315	460	561	1,350
Resistant seedlings	0 (0.00%)	0	0	0	0	0	0	0	0
2006									
Seedlings tested	10,040	3,366	798	1,582	865	816	1,717	350	546
Resistant seedlings	5 (0.05%)	2	0	1	0	0	0	0	2
Tetraploids	Overall	SE		SW		NE		NW	
		Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
2006									
Seedlings tested	6,200	436	907	546	897	593	678	1,012	1,134
Resistant seedlings	11 (0.17%)	0	2	0	0	0	0	0	9

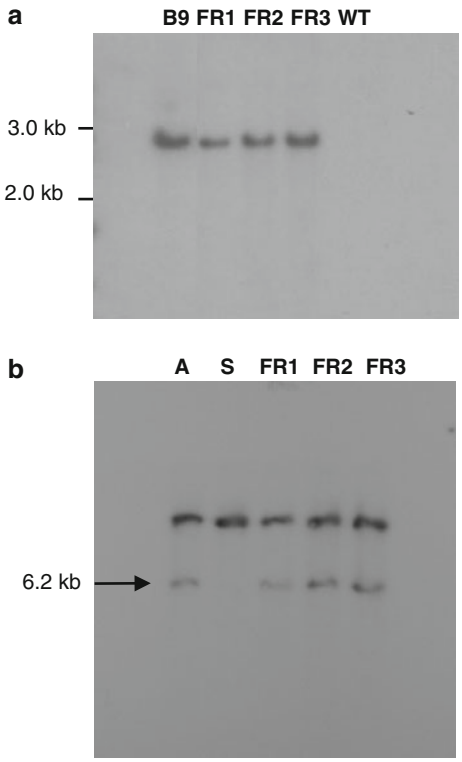

Fig. 4 Hybrid characterization using Southern blot and RFLP analysis. **a** Southern blot analyses showing *bar* gene integration of the B9 pollen parent and F_1 hybrids. Genomic DNA was digested with *Eco*RI which excises a 3.0-kb fragment in the plasmid pJFbar. Hybridization was performed using the full-length *bar* gene coding sequence as probe. The parent transgenic plant (B9) was used as the positive control and wild-type bahiagrass genomic DNA was the negative control. **b** RFLP marker linked to apospory in *Paspalum notatum*. The lower 6.2 kb band is apospory-specific in *P. notatum* (Martínez et al. 2003). Genomic DNA was digested with *Hin*dIII and hybridized with the apospory-specific rice probe C1069. The *arrow* indicates the apospory-specific restriction fragment 6,230 bp. *A* aposporous plant, *S* sexual plant

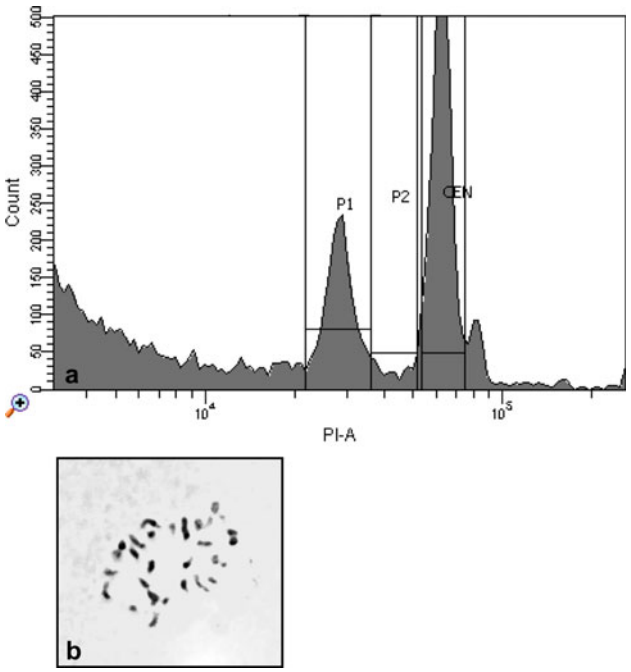

Fig. 5 Ploidy analyses in herbicide-resistant hybrids using flow cytometry and chromosome counting. **a** Flow cytometric analysis of PI-stained nuclei isolated from leaves of hybrid FR3 and chicken erythrocytes (CEN) as internal standard. PI-A value for peak P2 (corresponding to G1 peak for FR3) was detected at 30,050 (CV 9.8). The known tetraploid (4×) and diploid (2×) parent showed peaks at 41000 (CV 8.8) and 20,500 (CV 6.9), respectively. **b** Mitotic chromosomes of FR3 with $2n = 30$. Root tips were pretreated with 4-hydroxyquinoline followed by treatment with 95% ethanol:acetic acid (3:1 v/v). Observations were made at ×500 magnification

were evaluated for their phenotype and biomass accumulation under hydroponic growth conditions (Fig. 6a, b). The hybrids showed reduced vigor (biomass and seed head production) and drastically lower seed viability when

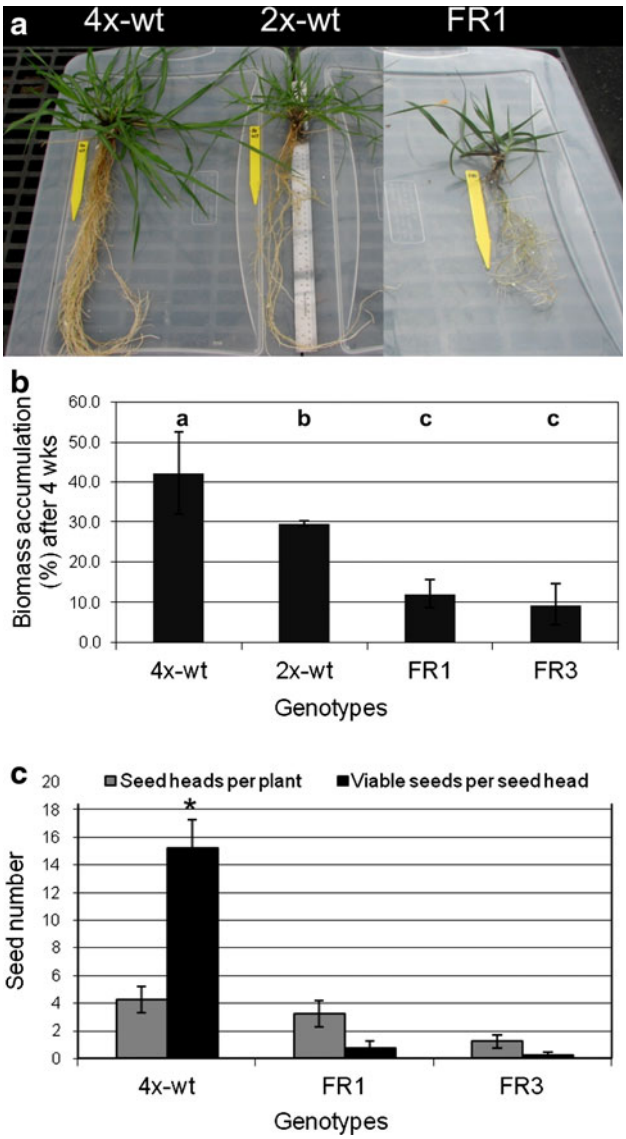

Fig. 6 Evaluation of phenotype and growth of hybrid lines when compared with tetraploid and diploid bahiagrass. **a** A representation of a hybrid plant after 4 weeks of hydroponics culture initiation when compared with tetraploid and diploid bahiagrass kept under the same conditions. **b** Biomass accumulation after 4 weeks of growth in hydroponics nutrient solution, for tetraploid (4×-wt), diploid (2×-wt) and the hybrid lines (FR1 and FR3). **c** Seed head production and seed viability in tetraploid (4×-wt) and the hybrid lines (FR1 and FR3). Statistical analysis of the data was done by comparing means using *t* test ($P < 0.05$). *Asterisk* indicates statistical difference at $P < 0.05$; *vertical bars* indicate the standard error

compared with wild-type bahiagrass. Biomass accumulation was highest for tetraploid wild type followed by diploid wild type, and the two hybrid lines ($P < 0.05$). Seed head production was not significantly different for the tetraploid wild type and hybrid lines FR1 and FR3. However, seed set in hybrids FR1 and FR3 was ≤1 per seed head (<1%, assuming ca. 100–120 spikelets per seed head are formed) and was significantly lower than a tetraploid wild type (Fig. 6c). Hybrid FR2 did not survive under greenhouse conditions, and FR4 and FR5 were less vigorous than FR1 and FR3 and had not reached reproductive maturity at the time of the experiment.

Discussion

Pollen-mediated gene flow is a critical component in the risk assessment of transgenic plants (Chandler and Dunwell 2008) and directly influences the formation of intra- and inter-specific hybrids as well as the content of transgenic seeds in non-transgenic crops.

Bahiagrass is one of the most abundant plants in the southeastern USA. Molecular improvement of bahiagrass for drought stress tolerance and improved turf quality has been reported recently (Agharkar et al. 2007; Zhang et al. 2007; James et al. 2008). Tetraploid bahiagrass is considered a pseudogamous obligate apomict (Burton 1948; Burton and Forbes 1960). A recent greenhouse study on pollen-mediated gene transfer indicated a low frequency of sexual seed production in tetraploid bahiagrass. Transgene flow from apomictic tetraploid to sexual diploids occurred at similar frequency as the tetraploid to tetraploid gene flow (Sandhu et al. 2009). Field data on pollen-mediated gene flow from apomictic bahiagrass are critically important because these frequencies are influenced by environmental conditions.

The highest gene transfer rate is expected if pollen donor and receptor are in close proximity and decreases dramatically over distance (Messeguer et al. 2004; Cunliffe et al. 2004). Wind direction is an important determinant of pollen and gene flow, but wind speed and turbulence may also have a large effect (Song et al. 2004; Giddings et al. 1997; Hoyle et al. 2007). Circular layouts, where transgenic plants are surrounded by pollen receptors allow capturing of gene transfer events in relation to wind direction (Messeguer et al. 2001). Our observations indicated highest gene transfer frequencies in the NW sector consistent with the prevailing wind direction. Nevertheless, this study demonstrates very low hybridization frequencies between transgenic and non-transgenic bahiagrass under field conditions. The observed gene transfer frequency from the transgenic tetraploid to non-transgenic diploid bahiagrass averaged between 0.00% in 2005 and 0.05% in 2006. Possible explanations for non-detectable gene transfer in 2005 when compared with the low, but detectable frequency in 2006 (0.05%) could be the higher relative humidity in 2005, which may have reduced anther dehiscence and pollen dispersal, or the reduced vigor of the plants during their establishment. In contrast to our observations, hybridization frequency up to 16% was reported for short distance gene flow from the facultative apomictic Kentucky bluegrass (Johnson et al. 2006).

Sexual hybridization between transgenic apomictic tetraploid and non-transgenic sexual diploid bahiagrass is likely prevented by the ploidy barrier which compromises endosperm formation in the absence of a 2:1 maternal/paternal ratio (Johnston et al. 1980). If a sexual diploid producing $2n$ central cell is pollinated with a $2n$ male gamete from the apomictic tetraploid (transgenic), this ratio is 1:1 and no viable seed is produced. However, incomplete penetrance of the endosperm balance number may occur due to random environmental events or by the process of endoreduplication within the polar nuclei (Johnston and Hanneman 1995). The triploid ($2n = 30$) or near triploid nature of the analyzed hybrid bahiagrass seedlings derived from diploid pollen receptors suggests that formation of a triploid from an 'n' female gamete and '$2n$' male gamete is a low frequency random event. Triploid formation by intra-specific diploid × apomictic tetraploid cross had a crossability frequency of 0.004% for *P. intermedium* and 0.015% for *P. brunneum* (Norrmann et al. 1994). The extremely low gene transfer frequency observed here from an apomictic tetraploid pollen donor to a sexual diploid pollen receptor is most likely due to the ploidy barrier. This conclusion is also supported by a comparison of gene flow frequency from transgenic, apomictic bahiagrass to a facultative apomictic bahiagrass genotype, which was 13 times higher than to a diploid sexual bahiagrass under identical greenhouse conditions (Sandhu et al. 2009). A field study with sexual diploid transgenic bahiagrass pollen donors could provide additional evidence for this conclusion. However, such an experiment will most likely not obtain regulatory approval.

Intra-specific crosses between sexual diploid genotype as male parent and facultative apomictic tetraploid genotypes as female parent of *P. notatum* have resulted in vigorous triploid progeny (Hanna and Burton 1986). In contrast, triploid hybrids obtained in the present study using sexual diploid genotype as female parent and obligate apomictic bahiagrass as pollen donor showed significantly reduced vigor accompanied by significantly lower biomass accumulation when compared with the diploid and tetraploid parents. Fertility and apomictic or sexual nature of triploid hybrids determine the potential for transgene introgression into natural wild-type populations of bahiagrass. Seed set in triploid hybrids was drastically reduced (<1%) when compared with OP diploid cultivar Tifton9 with average 39% seed set and OP tetraploid cultivar Argentine with average 36% seed set (Acuña 2006).

Apomictic and meiotic embryo sacs can coexist within the same plant or even with the same ovule (Koltunow 1993; Koltunow et al. 1995). The data presented here provide information about the degree of amphimixis present in tetraploid bahiagrass. Embryo-sac analysis of tetraploid bahiagrass Argentine has shown multiple apomictic embryo sacs in 95% of the ovules (Acuña et al. 2007). Transgenic lines from Argentine have produced uniform seed progeny without transgene segregation suggesting a high degree of apomixis (Sandhu and Altpeter 2008). Hybrid identification following pollen-mediated gene flow using a screenable marker-like herbicide resistance is the most accurate indicator of amphimixis in apomictic cultivars. The frequency of gene transfer between tetraploid, apomictic bahiagrass observed under field conditions (0.17%) and greenhouse conditions (0.16%; Sandhu et al. 2009) was very similar. The low gene transfer frequency between tetraploid, apomictic bahiagrass cultivars therefore confirms that the four evaluated tetraploid bahiagrass cultivars are highly apomictic, but also suggests that they should not be considered completely obligate apomicts.

Apomictic bahiagrass cultivars are known to produce a high percentage of viable pollen, which is essential for the formation of the endosperm by fusion with the central cells, termed pseudogamy (Burton and Forbes 1960; Quarin 1999). Quarin (1999) demonstrated that spikelets from apomictic bahiagrass that were emasculated and then isolated from pollen did not produce any seeds, while spikelets that were emasculated and then dusted with pollen of the same plant (selfing) produced seeds at normal frequency. In our experiments transgenic apomictic bahiagrass line B9 produced stainable pollen and produced seeds after selfing. Therefore, male sterility is not the cause of the observed extremely low gene flow rates. Tetraploid bahiagrass cultivars grown in the US show a very high frequency of apomixis (Acuña et al. 2007). Experimental evidence provided by Espinoza et al. (2002) indicates that aposporous embryo sacs reach maturity more precociously than meiotic sacs in facultative apomictic bahiagrass genotypes, resulting in only a remote chance that an egg cell will be fertilized during seed development under natural conditions. Therefore, apomixis restricts pollen-mediated gene flow to tetraploid apomictic pollen receptors. Our greenhouse experiment comparing gene flow rates to a well-characterized facultative apomictic bahiagrass genotype (2.07%) with gene flow rates to highly apomictic bahiagrass cultivars (0.16%; Sandhu et al. 2009) confirms this conclusion. In the present field study, we observed gene transfer from apomictic, tetraploid bahiagrass to non-transgenic diploid or tetraploid bahiagrass. However, despite the favorable conditions for gene flow the observed frequency is low as compared to the facultative apomict Kentucky bluegrass (Johnson et al. 2006) or other cross-pollinating grasses (e.g. Bae et al. 2008; Wang et al. 2004). Thus, both apomixis and ploidy barriers seem to suppress pollen-mediated gene flow drastically in apomictic, transgenic bahiagrass.

Acknowledgments We are grateful to the USDA-CSREES Biotechnology Risk Assessment Program for financial support. We appreciate Neal Benson (Genetics Institute, University of Florida) and Richard Fethiere (Agronomy Department, University of Florida) for their assistance with flow cytometry, Jeff Seib (Agronomy Department, University of Florida) for training Sukhpreet Sandhu in safe handling of radioisotopes, Carlos Alvarado (Agronomy Department, University of Florida) for excellent technical assistance and Hangning Zhang (Agronomy Department, University of Florida) for his help with molecular analysis. We like to thank Conrad Fafard Inc. Apopka, FL for the donation of Fafard potting mix.

References

Acuña CA (2006) Bahiagrass germplasm reproductive characterization, and breeding at the tetraploid level. MS Thesis, University of Florida, Gainesville, USA

Acuña CA, Blount AR, Quesenberry KH, Hanna WW, Kenworthy KE (2007) Reproductive characterization of bahiagrass germplasm. Crop Sci 47:1711–1717

Agharkar M, Lomba P, Altpeter F, Zhang H, Kenworthy K, Lange T (2007) Stable expression of *AtGA2ox1* in a low-input turfgrass (*Paspalum notatum* Flüggé) reduces bioactive gibberellin levels and improves turf quality in field conditions. Plant Biotech J 5:791–801

Aguado-Santacruz GA, Rascón-Cruz Q, Cabrera-Ponce JL, Martínez-Hernández A, Olalde-Portugal V, Herrera-Estrella L (2002) Transgenic plants of blue grama grass, *Bouteloua gracilis* (HBK) Lag. ex Steud., from microprojectile bombardment of highly chlorophyllous embryogenic cells. Theor Appl Genet 104:763–771

Altpeter F, James VA (2005) Genetic transformation of turf-type bahiagrass (*Paspalum notatum* Flüggé) by apomictic gene transfer. Intern Turfgrass Soc Res J 10:485–489

Bae TW, Vanjildorj E, Song SY, Nishiguchi S, Yang SS, Chandrashekhar T, Kang TW, Kim JI, Koh YJ, Lee J, Lee YE, Ryu KH, Lee HY, Park SY (2008) Environmental risk assessment of genetically engineered herbicide-tolerant *Zoysia japonica*. J Environ Qual 37:207–218

Bashaw EC, Funk CR (1987) Apomictic grasses. In: Fehr WR (ed) Principles of cultivar development, vol 2. Macmillan Publ. Co., New York, pp 40–82

Batra S, Kumar S (2003) *Agrobacterium*-mediated transient GUS gene expression in buffelgrass (*Cenchrus ciliaris* L.). J Appl Genet 44:449–458

Burson BL, Watson VH (1995) Bahiagrass, dallisgrass, and other *Paspalum* species. In: Barnes RF (ed) Forages: an introduction to grassland agriculture, 5th edn. 1. Iowa State University Press, Ames, pp 431–440

Burton GW (1942) Observations on the flowering habits of four *Paspalum* species. J Am Soc Agron 34:205–210

Burton GW (1946) Bahiagrass types. J Am Soc Agron 38:273–281

Burton GW (1948) The method of reproduction in common bahiagrass, *Paspalum notatum*. J Am Soc Agron 40:443–452

Burton GW, Forbes I (1960) The genetics and manipulation of obligate apomixis in common bahiagrass (*Paspalum notatum* Flüggé). In: Skilmore CL (ed) Proceedings international Grassland congress, 8th, Reading, England. 11–21 July 1960. Alden Press, Oxford, pp 66–71

Chambliss CG, Adjei MB (2006) Bahiagrass. EDIS SS-AGR-36

Chandler S, Dunwell JM (2008) Gene flow, risk assessment and the environmental release of transgenic plants. Crit Rev Plant Sci 27:25–49

Chapman MA, Burke JM (2006) Letting the gene out of the bottle: the population genetics of genetically modified crops. New Phytol 170:429–443

Cunliffe KV, Vecchies AC, Jones ES, Kearney GA, Forster JW, Spangenberg GC, Smith KF (2004) Assessment of gene flow using tetraploid genotypes of perennial ryegrass (*Lolium perenne* L.). Aust J Agric Res 55:389–396

Dalton SJ, Bettany AJE, Bhat V, Gupta MG, Bailey K, Timms E, Morris P (2003) Genetic transformation of *Dichanthium annulatum* (Forssk)—an apomictic tropical forage grass. Plant Cell Rep 21:974–980

Espinoza F, Pessino SC, Quarin CL, Valle EM (2002) Effect of pollination timing on the rate of apomictic reproduction revealed by RAPD markers in *Paspalum notatum*. Ann Bot 89:16–170

Forbes I, Burton GW (1961) Cytology of diploids, natural and induced tetraploids and intraspecific hybrids of bahiagrass, *Paspalum notatum* Flüggé. Crop Sci 1:402–406

Gao C, Jiang L, Folling M, Han L, Neilson KK (2006) Generation of large numbers of transgenic Kentucky bluegrass (*Poa pratensis* L.) plants following biolistic gene transfer. Plant Cell Rep 25:19–25

Gates RN, Quarin CL, Pedreira CGS (2004) Bahiagrass. In: Moser LE, Burson BL, Sollenberger LE (eds) *Warm-Season (C4) grasses*, Agronomy monograph no 45 s. ASA, CSSA, SSSA, Madison

Giddings GD, Sackville-Hamilton NR, Hayward MD (1997) The release of genetically modified grasses. Part 2: the influence of wind direction on pollen dispersal. Theor Appl Genet 94:1007–1014

Gould FW (1966) Chromosome numbers of some Mexican grasses. Can J Bot 44:1683–1696

Ha CD, Lemaux PG, Cho MJ (2001) Stable transformation of a recalcitrant Kentucky bluegrass (*Poa pratensis* L.) cultivar using mature seed-derived highly regenerative tissues. Vitro Cell Dev Biol Plant 47:6–11

Hanna W (1995) Use of apomixis in cultivar development. Adv Agron 54:333–350

Hanna WW, Burton GW (1986) Cytogenetics and breeding behavior of an apomictic triploid in bahiagrass. J Hered 77:457–459

Hoyle M, Hayter K, Cresswell JE (2007) Effect of pollinator abundance on self-fertilization and gene flow: application to GM canola. Ecol Appl 17:2123–2135

James VA, Neibaur I, Altpeter F (2008) Stress inducible expression of *DREB1A* transcription factor from xeric *Hordeum spontaneum* L. in turf and forage grass (*Paspalum notatum* Flüggé) enhances abiotic stress tolerance. Transgenic Res 17:93–104

Johnson PG, Larson SR, Anderton AL, Patterson JT, Cattani DJ, Nelson EK (2006) Pollen-mediated gene flow from Kentucky bluegrass under cultivated field conditions. Crop Sci 46:1990–1997

Johnston SA, Hanneman RE Jr (1995) The genetics of triploid formation and its relationship to endosperm balance number in potato. Genome 39:314–321

Johnston SA, den-Nijs TM, Peloquin SJ, Hanneman RE Jr (1980) The significance of genic balance to endosperm development in interspecific crosses. Theor Appl Genet 57:5–9

Jorgensen RB, Hauser T, D'Hertefeldt T, Andersen NS, Hooftman D (2009) The variability of processes involved in transgene dispersal—case studies from Brassica and related genera. Environ Sci Pollut Res 16:389–395

Koltunow AM (1993) Apomixis: embryo sacs and embryos formed without meiosis or fertilization in ovules. Plant Cell 5:1425–1437

Koltunow AM, Bicknell RA, Chaudhury AM (1995) Apomixis—molecular strategies for the generation of genetically identical seeds without fertilization. Plant Physiol 108:1345–1352

Kumar J, Shukla SM, Bhat V, Gupta S, Gupta MG (2005) In vitro plant regeneration and genetic transformation of *Dichanthium annulatum*. DNA Cell Biol 24:670–679

Martínez EJ, Hopp HE, Stein J, Ortiz JPA, Quarin CL (2003) Genetic characterization of apospory in tetraploid *Paspalum notatum* based on the identification of linked molecular markers. Mol Breed 12:319–327

Messeguer J, Fogher C, Guiderdoni E, Marfa V, Catala MM, Bald G, Mele E (2001) Field assessments of gene flow form transgenic to cultivated rice (*Oryza sativa* L.) using herbicide resistance gene as tracer marker. Theor Appl Genet 103:1151–1159

Messeguer J, Marfa V, Catala MM, Guideroni E, Mele E (2004) A field study of pollen-mediate gene flow from Mediterranean GM rice to conventional rice and the red rice weed. Mol Breed 13:103–112

Messeguer J, Penas G, Ballester J, Bas M, Serra J, Salvia J, Palaudelmas M, Mele E (2006) Pollen-mediated gene flow in maize in real situations of coexistence. Plant Biotech J 4:633–645

Murray MG, Thompson WF (1980) Rapid isolation of high molecular-weight plant DNA. Nucleic Acids Res 8:4321–4325

Norrmann GA, Bovo OA, Quarin CL (1994) Post-zygotic seed abortion in sexual diploid x apomictic tetraploid intraspecific *Paspalum* crosses. Aust J Bot 42:449–456

Pozzobon MT, Valls JFM (1997) Chromosome number in germplasm accessions of *Paspalum notatum* (Gramineae). Braz J Genet 20:29–34

Quarin CL (1999) Effect of pollen source and pollen ploidy on endosperm formation and seed set in pseudogamous apomictic *Paspalum notatum*. Sex Plant Reprod 11:331–335

Quarin CL, Burson BL, Burton GW (1984) Cytology on intra- and inter-specific hybrids between two cytotypes of *Paspalum notatum* and *P.* Cromyorrhizon. Bot Gaz 145:420–426

Quarin CL, Espinoza F, Martínez EJ, Pessino SC, Bovo OA (2001) A rise of ploidy level induces the expression of apomixis in *Paspalum notatum*. Sex Plant Reprod 13:243–249

Rong J, Lu BR, Sing Z, Su J, Snow AA, Zhang X, Sun S, Chen R, Wang F (2007) Dramatic reduction of crop-to-crop gene flow within a short distance from transgenic rice fields. New Phytol 173:346–353

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Sandhu S, Altpeter F (2008) Co-integration, co-expression and inheritance of unlinked minimal transgene expression cassettes in apomictic turf and forage grass (*Paspalum notatum* Flüggé). Plant Cell Rep 27:1755–1765

Sandhu S, Altpeter F, Blount AR (2007) Apomictic bahiagrass expressing the *bar* gene is highly resistant to glufosinate under field conditions. Crop Sci 47:1691–1697

Sandhu S, James V, Quesenberry K, Altpeter F (2009) Risk assessment of transgenic apomictic tetraploid bahiagrass, cytogenetics, breeding behavior and performance of intra-specific hybrids. Theor Appl Genet 119:1383–1395

Saura F (1948) Cariologia de gramineas en Argentina. Rev Fac Agron Vet Buenos Aires 12:51–67

Smith RL, Grando MF, Li YY, Seib JC, Shatters RG Jr (2002) Transformation of Bahiagrass (*Paspalum notatum* Flüggé). Plant Cell Rep 20:1017–1021

Song Z, Lu BR, Zhu YG, Chen J (2004) Pollen flow of cultivated rice measured under experimental conditions. Biodivers Conserv 13:579–590

Stein J, Quarin CL, Martínez EJ, Pessino SC (2004) Tetraploid races of *Paspalum notatum* show polysomic inheritance and preferential chromosome pairing around the apospory-controlling locus. Theor Appl Genet 109:186–191

Wang ZY, Lawrence A, Hopkins A, Bell J, Scott M (2004) Pollen-mediated transgene flow in the wind-pollinated grass species tall fescue (*Festuca arundinaceae* Schreb.). Mol Breed 14:47–60

Wang F, Yuan QH, Shi L, Qian Q, Liu WG, Kuang BG, Zeng DL, Liao YL, Cao B, Jia SR (2006) A large-scale field study of transgene flow from cultivated rice (*Oryza sativa*) to common wild rice (*O. rufipogon*) and barnyard grass (*Echinochloa crusgalli*). Plant Biotech J 4:667–676

Zhang H, Lomba P, Altpeter F (2007) Improved turf quality of transgenic bahiagrass (*Paspalum notatum* Flüggé) constitutively expressing the ATHB16 gene, a repressor of cell expansion. Mol Breed 20:415–423