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ORIGINAL PAPER

Risk assessment of transgenic apomictic tetraploid bahiagrass, cytogenetics, breeding behavior and performance of intra-specific hybrids

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Abstract Pollen-mediated gene transfer from stress tolerant or herbicide-resistant transgenic plants may cause environmental or agronomic problems. Apomictic seed production found in some bahiagrass cultivars may serve as a natural transgene containment system. Under greenhouse conditions, the average gene transfer frequency from an herbicide-resistant apomictic tetraploid to a population of sexual diploid bahiagrass genotypes or apomictic tetraploid bahiagrass was 0.16% when the transgenic pollen donor was placed at 0.5–1.5 m distance from the non-transgenic pollen receptors. The herbicide-resistant hybrids were characterized for transgene integration, expression and ploidy, by Southern blot analysis, immuno-chromatography and flow cytometry, respectively. Hybrids resulting from open pollination of non-transgenic diploid female plants with transgenic tetraploid male plants were triploids or near-triploids, with $2n = 26$–34. These hybrids displayed a wide range of phenotypic variability, including some non-persistent or non-flowering dwarf-type hybrids with good vigor, or hybrids with vegetative growth similar to non-transgenic plants, but with significantly reduced seed set. Non-flowering aneu-triploids with good vigor/field performance will provide the highest level of transgene containment. Embryo sac analysis of pollinated spikelets confirmed a high proportion of aborted ovules. An apospory-linked RFLP marker was detected in 13 of the 15 near-triploid hybrids. All flowering aneuploid hybrids displayed significantly reduced seed set, and none of the sexual near-triploid hybrids produced any seeds. All tetraploid gene transfer events carried the apospory-linked RFLP marker, suggesting that despite the presence of the aposporus locus, a low degree of sexuality co-exists in apomictic tetraploid cultivars. Thus, tetraploid apomictic bahiagrass does not provide complete transgene containment, although intra-specific gene transfer is drastically reduced compared to sexually reproducing perennial grasses.

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Introduction

Bahiagrass (*Paspalum notatum* Flugge) is a warm-season perennial grass indigenous to South America and has become naturalized in the sub-tropical climate of the southeastern USA (Gates et al. 2004). Its popularity as a forage and turf species stems from attributes such as low maintenance requirements, and good tolerance to drought and most diseases and pests. The most dominant cytotypes in the South American habitat are tetraploid ($2n = 4x = 40$), which produce seed asexually through apomixis. Naturally occurring sexual tetraploid plants have not been found in bahiagrass (Espinoza et al. 2006). Diploid races of bahiagrass represent the dominant cytotype found in the southeastern states of USA. They are wind pollinated, allogamous, self-incompatible and inhabit limited areas in its native habitat (Daurelio et al. 2004).

Apomixis in *P. notatum* is characterized by apospory, where unreduced embryo sacs develop from nucellar

somatic cells. Sexual or apomictic plants can be differentiated on the basis of structure, number of embryos sacs per ovule and orientation of egg cell in the embryo sac. A sexual plant has a characteristic single embryo sac, with egg cell towards the micropylar end and antipodals towards the chalazal end. Apomictic plants show multiple embryo sacs with an unreduced egg cell and central nuclei, but lack the antipodal cells (Ortiz et al. 1997; Quarin et al. 2001). There are two hypotheses that explain the complete absence or lack of expression of apomixis in diploid cytotypes. The first hypothesis, proposed by Nogler (1984), is based on the assumption that monoploid ($n = x$) gametes cannot transfer the apomictic trait. Apomixis is thus a monogenic dominant trait that can only be transmitted in the heterozygous state through diploid or polyploid gametes. Apospory in *P. notatum* and *P. simplex* is inherited as a single dominant gene with tetrasomic inheritance and incomplete penetrance. A pleiotropic lethal effect is proposed to prevent transmission of apospory through monoploid gametes (Martinez et al. 2001; Caceres et al. 2001). The second hypothesis, proposed by Mogie (1988), assumes a dosage requirement for the expression of apomixis. The absence of apomixis in diploids could be caused by low expression rather than non-transmission of apomictic genes (Quarin et al. 2001). Triploid hybrids obtained from crosses between sexual $2x$ and apomictic $4x$ genotypes may allow deeper insight into the inheritance and expression of apomixis in *P. notatum* (Martinez et al. 2007). Molecular markers, such as random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphism (AFLPs) and restriction fragment length polymorphism (RFLPs), completely linked to apospory have been described (Ortiz et al. 1997; Stein et al. 2004; Martinez et al. 2003). The RFLP rice C1069 locus is a codominant marker mapping to the telomeric long arm of chromosome 12. It is considered to be completely linked to apospory in *P. simplex* and *P. notatum* (Pupilli et al. 2001; Martinez et al. 2003).

Apomixis allows fixation of hybrid vigor while maintaining purity of superior genotypes. The apomictic bahiagrass cultivar 'Argentine' is a preferred target for genetic transformation, since its apomictic seed production would result in uniform progeny, and also potentially reduce the risk of unintended gene dispersal by pollen. It is also a commercially important cultivar; however, genetic improvement through traditional breeding has been very difficult due to the lack of female meiotic recombination during seed production. Smith et al. (2002) first reported the recovery of transgenic bahiagrass plants after biolistic introduction of the *bar* gene into the apomictic accession 'Tifton 7'. Altpeter and James (2005) described a highly efficient biolistic transformation protocol for the apomictic cultivar Argentine. This protocol has now been successfully used to introduce a number of important traits into Argentine bahiagrass, including herbicide resistance (Sandhu et al. 2007), abiotic stress tolerance (James et al. 2008) and improved turf quality (Agharkar et al. 2007; Zhang et al. 2007). Transgenic, herbicide-resistant bahiagrass plants of the sexual, diploid cultivar 'Pensacola' (Gondo et al. 2005) and insect resistant plants of the diploid cultivar 'Tifton 9' (Luciani et al. 2007) have also been reported.

Pollen-mediated gene transfer from genetically modified (GM) plants may cause environmental or agronomic problems. Transgenic crops can be broadly categorized into high, medium and low risk crops based on the reproductive biology, mode of pollination, location of the crop and production area (Stewart et al. 2003). Crop species that are genetically distinct, and where introgression is limited to the geographic center of diversity, are very low risk crops. Wind cross-pollinated species, with a prevalence of related species in the same genus, are considered high risk crops. Tetraploid to diploid gene flow may occur through the formation of triploids. Naturally occurring triploids have been found near the center of diversity for *P. notatum* (Quarin et al. 1989; Tischler and Burson 1995). Because of the ploidy barrier, very low gene transfer frequencies are expected from tetraploid to diploid bahiagrass. Detailed reproductive and cytogenetic characterization of hybrid plants will add valuable information for risk assessment of transgenic bahiagrass.

Herbicide resistance as a screenable marker is useful in the efficient identification of rare pollen-mediated gene transfer events. It could also assist in the reproductive classification of apomictic tetraploids. The ovule clearing technique has been used to differentiate apomictic and meiotic embryo sacs (Young et al. 1979; Quarin et al. 1982; Acuna 2006). However, whether meiotic embryo sacs present in apomictic plants form viable seeds is unclear. Seed progeny analysis is hence important to determine the frequency of functional apomixis or amphimixis (Ozias-Akins 2006). Any sexual reproduction in a heterozygous apomictic plant will produce 'off-type' individuals. A quantitative assessment of the degree of sexuality versus apomixis can be estimated by the use of a dominant phenotypic marker. Flow cytometry is a widely used technique for rapid detection of DNA ploidy (Valkonen 1994; Taliaferro et al. 1997; Dolezel et al. 1997; Roux 2003; Dolezel et al. 2007). It has been applied for large-scale comparative analyses of genome size, reproductive biology, taxonomic identification and delineation (reviewed by Kron et al. 2007; Ochatt 2008). The flow cytometric seed screen (FCSS, Matzk et al. 2000) is an alternative method to establish an apomictic or amphimictic mode of reproduction.

The present study evaluates the gene flow frequency between apomictic tetraploid and sexual diploid or apomictic tetraploid bahiagrass, and the nature of hybrids produced, including transgene integration pattern, ploidy, fertility, phenotypic and fitness characteristics.

Materials and methods

Pollen donor

The pollen donor for this gene flow study was a transgenic herbicide-resistant bahiagrass line (B9) derived from the apomictic tetraploid cv. Argentine. Embryogenic bahiagrass calli were transformed with the plasmid pJFbar using biolistic transformation. Plasmid pJFbar contains the *bar* gene (Thompson et al. 1987) under control of the constitutive maize ubiquitin promoter and first intron (Christensen and Quail 1996) and the CaMV 35S 3′UTR (Dixon et al. 1986). Transgenic calli and regenerating shoots were selected for herbicide resistance by inclusion of 3 mg l^{-1} bialaphos (Phytotechnologies Inc., Shawanee Mission, KS, USA) in the culture medium. For Southern blot analysis, genomic DNA from line B9 was digested using *Bgl*II and hybridized with a radio-labeled probe from the full-length coding region of the *bar* gene, according to standard procedures (Sambrook and Russell 2001). Herbicide resistance of B9 was confirmed by application of 0.3% glufosinate (the recommended application rate of the herbicide Ignite,with 18.1% glufosinate ammonium as active ingredient, AgrEvo Inc., Wilmington, DE, USA).

Viable pollen is required for endosperm formation in Argentine seeds. Endosperm formation following complete exclusion of pollen from other plants (selfing) indicated viable pollen production of B9. The emerging inflorescences were bagged in a glassine pollination bag to exclude foreign pollen. The bags were shaken every morning (between 6:30 and 7:30 a.m.) to ensure pollination. The viability of selfed seeds, presumably of apomictic origin, was confirmed by germination. Pollen stainability (described under hybrid characterization) and apomictic seed production in B9 was also observed. Uniform transgene transmission to 30 open-pollinated seed progeny was tested by the application of 0.3% glufosinate using the original parent B9 as a positive control (PC) and a non-transgenic bahiagrass plant as a negative control (NC).

Open-pollination and seed head collection

Non-transgenic bahiagrass plants were allowed to open-pollinate (OP) with transgenic bahiagrass B9 placed at a distance of 0.5–1.5 m. Synchronously flowering transgenic pollen donor plants and non-transgenic pollen receptor plants were selected for pollination experiments. A flex duct system in the greenhouses provided continuous airflow (6–9 m s^{-1}). Irrigation was provided once a day by an automated ebb and flow irrigation system. The plants were maintained at 28/23°C day $night^{-1}$ temperature and natural photoperiod. This greenhouse facility and the standard operating procedures for handling of transgenic plants were approved and monitored by the institution's biosafety committee.

Equal numbers of transgenic B9 pollen donor plants (10) were used in all experiments to generate similar pollen sources. The gene flow from transgenic apomictic bahiagrass to non-transgenic bahiagrass was evaluated in five sets in different greenhouses:

(1) Pollen-mediated gene transfer from transgenic apomictic tetraploid to a population of 30 different non-transgenic sexual diploid bahiagrass genotypes, (2) pollen-mediated gene transfer from transgenic apomictic tetraploid to 30 clonal plants of a single diploid genotype (clone 1), (3) pollen-mediated gene transfer from transgenic apomictic tetraploid to 30 clonal plants of a second single diploid genotype (clone 2),[1] (4) pollen-mediated gene transfer from transgenic apomictic tetraploid to three non-transgenic highly apomictic tetraploids (cv. Argentine, cv. Paraguay 22 and the experimental hybrid Tifton 7); and (5) pollen-mediated gene transfer from transgenic apomictic tetraploid to a non-transgenic facultative apomictic tetraploid accession with a high degree of sexuality (determined by embryo sac analysis). This genotype is referred to as 'facultative apomictic accession' for simplicity.

Inflorescences were tagged at anthesis to monitor synchronous flowering of transgenic pollen donors and non-transgenic pollen receptors. Seed head tagging was color coded for each week and the anthesis date was recorded on each tag. Seed heads were bagged in glassine pollination bags 10 days after pollination, to prevent seed loss, and harvested 30 days later. Seeds from the three different apomictic cultivars in the fourth experiment set were harvested separately. Seeds were air-dried and stored in plastic containers prior germination. Seed head collection continued until the end of flowering.

Screening of seed progeny

Seeds were germinated following acid treatment to break seed dormancy. Seedlings were grown in germination flats containing Fafard 2 potting mix (Fafard Inc., Apopka FL, USA) and were maintained in the greenhouse at

[1] Clonal plants for the second and third experiment set were generated by clonal propagation of a single seedling of cultivar Pensacola in liquid culture media containing 4.3 g l^{-1} MS salts with vitamins (Murashige and Skoog 1962), 30 g l^{-1} sucrose and 0.3 mg l^{-1} 6-benzylaminopurine (BAP).

28/23°C day night^{-1} temperature and natural photoperiod. The herbicide concentration was optimized to identify transgenic hybrids, even if they expressed the transgene at a low level. A concentration of 0.14% glufosinate (Ignite®, ArgEvo, Wilmington, DE, USA) was applied to seedlings until runoff at 6 weeks after germination. This is the lowest concentration of glufosinate ammonium which still produced 80–100% necrosis in non-transgenic bahiagrass. Herbicide-resistant seedlings (putative F_1 hybrids) were scored for necrosis 3 weeks after glufosinate application.

Hybrid confirmation

Transgene expression

Protein extracts of all herbicide-resistant putative F_1 hybrid plants obtained from herbicide screening were tested for the presence of the *bar* gene product, phosphinothricin acetyl transferase (PAT), using the immuno-chromatographic lateral membrane Quick Stix™ (LibertyLink®/bar, Envirologix, Portland, ME, USA). Total protein was extracted from 300 mg leaf tissue using 150 µl extraction buffer (supplied with the kit). Protein extract from non-transgenic bahiagrass was used as a NC and the transgenic parent B9, known to express PAT was used as a PC.

Transgene integration

Integration of the *bar* gene in all herbicide-resistant hybrids confirmed by the Stix assay was analyzed by Southern blotting. Genomic DNA was extracted from fresh leaf tissue according to the CTAB method (Murray and Thompson 1980) and digested with *Bgl*II. Standard procedures for DNA blotting and hybridization were followed (Sambrook and Russell 2001). The full-length coding sequence of the *bar* gene was radio-labeled with [^{32}P] dCTP using the Prime-a-gene® labeling system (Promega, U.S., Madison, WI, USA) and used as a probe.

Hybrid characterization

Flow cytometric determination of ploidy

Hybrids R1, R2, R3, R4, R5, R6, R8, R12, R14, R17, R18, R19, R20 and R21 (from OP diploid bahiagrass), the transgenic parent B9 and hybrid R26 (from OP tetraploid bahiagrass) were characterized for DNA ploidy using a CyFlow® space Flow Cytometry System (Partec North America Inc., Mt. Laurel, NJ, USA). Shoot apical meristem (ca. 5 mm section) was macerated in 500 µl nuclei extraction buffer (CyStain® PI Absolute P; North America Inc., Mt. Laurel, NJ, USA) for 60 s using a razor blade. The material was filtered through a 50 µm filter followed by the addition of a drop of chicken erythocytes (as internal standard; Biosure Inc., Grass Valley, CA, USA) and 2 ml propidium iodide (PI) stain. The filtrate was incubated for 1 h in the dark at 4°C before reading. The analyzer was arbitrarily calibrated using a known tetraploid bahiagrass and a known diploid bahiagrass. Three biological replicates for each sample were performed. The DNA index was calculated as the ratio of sample fluorescence to the fluorescence of an internal standard run in parallel with each sample.

Karyotypic analysis using root-tip preparations

Root-tip mitotic chromosomes were evaluated for several F_1 hybrids (including R1, R2, R3, R4, R5 and R6) to confirm the flow-cytometric ploidy determination. Cleaned healthy roots (ca. 5 cm) were pretreated for 4 h with 8-hydroxyquinoline, followed by overnight treatment with the fixative (95% ethanol: acetic acid, 3:1 v/v). Fixed material was stored in 70% ethanol. For root-tip preparations, a 2 mm section of the root-tip (excluding the root cap) was excised and chopped in 45% acetic acid, followed by addition of aceto-orcein stain. A cover slip was carefully placed and gently tapped to spread the cells. The material was allowed to stain for 30 min. Chromosomes were counted at 500× or 1,000× magnification using a compound microscope (Am Scope, Model T400A; American Scope, USA).

Dry matter, seed production and viability

Individual tillers of hybrids from OP diploids R1, R2, R3, R4, R5, R8, R17, R18, R19, R20, R21, and R27, a hybrid from OP tetraploid R26, a non-transgenic tetraploid (NT-4x) and a diploid bahiagrass plant (NT-2*x*) were initiated in hydroponic growth solution, consisting of 1.2% boost and 0.3% grow (Technaflora Plant Products, Port Coquitlam, BC, Canada), pH 6.0. Roots were fully submerged into aerated nutrient solution with approximately 80% oxygen saturation. Multiple replications (tillers) were organized in a completely randomized block design. After 4 weeks, four plantlets (initiated from single tillers) from each hybrid and the diploid and tetraploid bahiagrass plants were transplanted into Fafard 2 potting mix (Fafard Inc., Apopka FL) and allowed to grow for 10 weeks under natural photoperiod (varying from 12 to 15 h day length) and 28 ± 2°C temperature. Seed heads produced during this time were collected and used to determine seed set. Seed heads were thrashed and seed set was calculated as the number of spikelets containing caryopses per seed head. After seed heads were removed, the remaining above ground biomass was harvested and dried at 50°C for 48 h, after which dry weight was recorded. Data were analyzed

using PROC ANOVA of SAS version 9.1 (SAS Institute). Means were compared by the *t* test statistic (LSD, $P < 0.05$). Seed germination was checked by imbibing seeds in water overnight, followed by dissection of lemma and palea. Germinated seedlings were maintained in a growth chamber with 12 h photoperiod, 28/23°C day - night^{-1} temperature.

Embryo sac analysis and pollen viability

Inflorescences produced on three large greenhouse grown plants of hybrids R1, R3, R4, R5 and R6, NT-4*x*, and NT-2*x* were used for embryo sac and pollen viability. Inflorescences were collected at anthesis and fixed in FAA (18 ethanol 70%: 1 formaldehyde 37%: 1 glacial acetic acid) for 24 h. Pistils were dissected from the spikelets and prepared following the ovule clearing technique of Young et al. (1979). Ovules were observed under a differential interference contrast (DIC) microscope (Nikon®, Melville, NY, USA). A minimum of 20 ovules per plant were analyzed. Meiotic embryo sacs were characterized by a single embryo sac containing the egg cell and the central nuclei at the micropylar end, and a mass of antipodals at the chalazal end. Apomictic embryo sacs were differentiated by the presence of multiple embryo sacs, and also by the position of the egg cell. Pollen viability was based on pollen staining. The term 'pollen stainability' has been used interchangeably with 'pollen viability' and only refers to the percentage of stained pollen, indicating viable pollen DNA. However, pollen stainability only represents a normal pollen formation, but does not necessarily translate to pollen viability as many stainable pollen grains may not germinate. A clean, glass slide containing a drop of aceto-orcein stain was dusted with pollen from a dehiscing inflorescence. A cover slip was gently placed on top and observations of pollen viability were recorded using a microscope (Amercian Scope, USA) fitted with line transects. Viable pollen absorbed the stain and appeared dark red, while non-viable pollen was white or faint pink.

RFLP marker analysis

RFLP analysis of hybrids R1, R2, R3, R4, R5, R7, R8, R12, R17, R18, R20, R21, R22 and R14, originating from OP sexual diploids, and hybrids R23, R25, R26, R40, R41, R42, R43, R44, R45, R50, R52 and R54, orginating from OP apomictic tetraploids, was performed. The procedure, as described by Martinez et al. (2003),was followed with minor modifications. Following complete digestion with restriction enzyme *Eco*RI, the samples were electrophoresed in a 1.5% w/v agarose gel, 1×TAE, overnight at 20 V. DNA was blotted onto nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using 20× SSC, following the neutral transfer procedure. The C1069 probe (Accession no. D15675) was amplified from rice (*Oryza sativa* cv. Japonica) genomic DNA using primers designed from the sequence information in the NCBI database. The PCR product was purified using the Qiagen PCR purification kit (Qiagen Inc., Valencia, CA, USA). Successful amplification of C1069 was confirmed by sequence analysis (MWG-Biotech Inc., High Point NC). Following sequence confirmation, the C1069 probe was radio-labeled with [^{32}P] dCTP using the Prime-a-gene labeling system (Promega U.S., Madison, WI, USA). The hybridization buffer containing labeled probe and denatured herring sperm DNA (1 μg μl^{-1}) were incubated at 65°C in a hybridization oven. Washing was performed at 65°C and included the following steps: quick wash using 2× SSC; 20 min low stringency wash using 2× SSC, 0.5% SDS; 20 min high stringency wash using 0.2× SSC, 0.1% SDS. The hybridized membrane was placed in an autoradiographic cassette (FisherBiotech® Four Square Cassettes, Fisher Scientific, Pittsburgh, PA, USA) along with an autoradiographic film (Kodak Biomax MS autoradiography). Following a 48 h exposure period at −80°C, the autoradiographic film was developed.

Results

Transgenic pollen source and synchrony of flowering

Single copy *bar* gene integration in pollen donor line B9 was detected by Southern blot analysis (shown in Fig. 1b). Dark red stained pollen suggested the production of viable pollen in the transgenic line B9. Transgenic line B9 did not differ from non-transgenic bahiagrass in growth and seed set. This line produced viable seeds under open and self pollination. All 30 seed-derived progeny plants were resistant to 0.3% glufosinate (data not shown).

Weekly tagging of pollinating seed heads facilitated monitoring of synchronous flowering of transgenic and non-transgenic plants and identification of the peak flowering period. Seeds formed during the peak flowering period were selected for progeny analysis. For each experiment, there were at least 30 pollinating seed heads per genotype and replication, during this peak flowering period.

Pollen-mediated gene transfer from transgenic tetraploid to diploid bahiagrass

The first greenhouse experiment, with a population of diploid genotypes as pollen receptors resulted in 0.16% gene transfer frequency from the transgenic apomictic pollen donor (13 herbicide-resistant hybrids out of 8,330

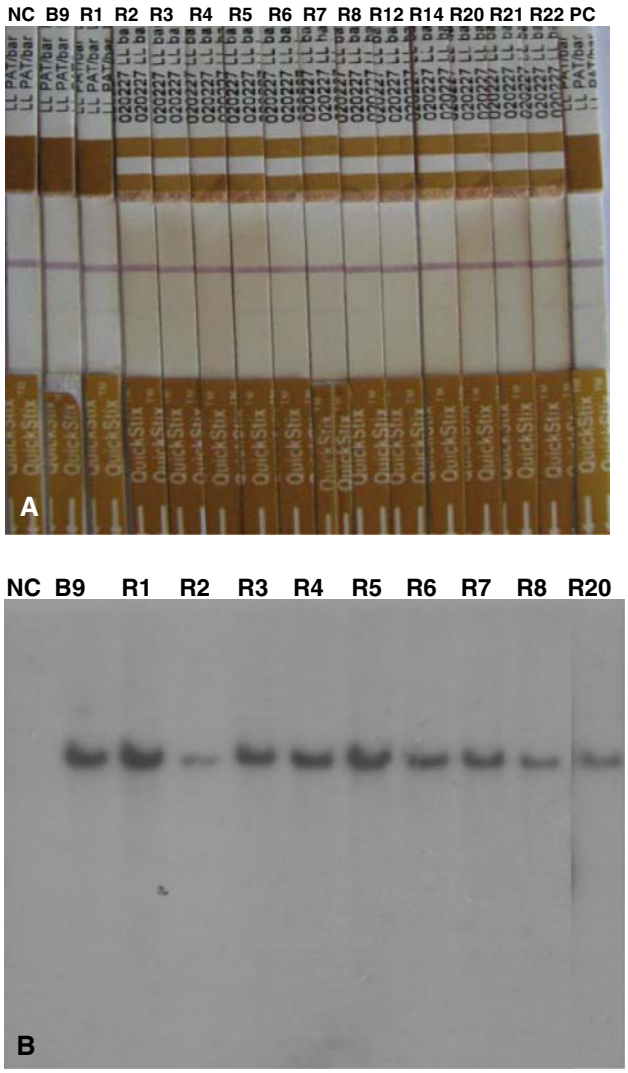

Fig. 1 Transgene expression and integration in representative F_1 hybrids obtained from sexual diploid pollen receptors open-pollinated with transgenic apomictic tetraploid bahiagrass. **a** Immuno-chromatographic determination of *bar*/PAT expression in the transgenic pollen parent B9 and F_1 hybrid plants using the Libertylink® Stix Test. The top band is common to all samples and indicates functionality of the stix assay. The lower PAT-specific band confirms the presence of PAT in the protein extract. **b** Southern blot image showing *bar* gene integration in the transgenic pollen parent B9 and F_1 hybrids (R-). Genomic DNA digested with *Bgl*II was used for blotting. The probe represented the full-length coding region of the *bar* gene. In both A and B, *NC* non-transgenic bahiagrass, *PC* transgenic pollen parent B9, hybrids R1-R7 originating from a population of sexual diploid pollen receptors, R8 and R20 originating from a clonal diploid genotype

seedlings, Table 1). The frequency of gene transfer to diploid genotypes of clonal origin differed according to the individual clone. Clone 1 showed a hybridization rate of 3.6% (12 herbicide-resistant hybrids out of 332 seedlings), whereas clone 2 did not produce any hybrids (none out of 5,209 seedlings, Table 1). It was also observed that clone 1 (1,900 seeds) formed less seed from the same number of plants than clone 2 (13,400 seeds).

Pollen-mediated gene transfer from transgenic tetraploid to wild-type tetraploid bahiagrass

The fourth greenhouse experiment, with OP tetraploid cultivars as pollen receptors, yielded ten herbicide-resistant hybrids out of 6,297 seedlings (0.16%, Table 2). Amongst the highly apomictic tetraploids, Paraguay 22 showed the highest hybridization frequency (0.32%), producing five herbicide-resistant hybrids from 1,559 seedlings. Argentine bahiagrass showed 0.11% hybridization frequency, yielding four herbicide-resistant hybrids out of 3,669 seedlings, while Tifton 7 progeny produced one herbicide-resistant hybrid out of 1,062 seedlings (0.09% hybridization frequency). Paraguay 22 also produced the most seed heads, followed by Argentine, and Tifton 7 (data not shown). The facultative apomictic accession used as a pollen receptor in the fifth greenhouse experiment produced 15 herbicide-resistant hybrids out of 724 seedlings (2.7%, Table 2).

Hybrids—characterization of transgene integration and expression

Herbicide-resistant seedlings (putative F_1 hybrids) were confirmed for PAT activity with the lateral membrane flow immuno-chromatography assay (a representative image is shown in Fig. 1a). All herbicide resistant confirmed F_1 hybrids were found to harbor a single functional copy of the *bar* gene, identical to the pollen parent B9, by Southern blot analysis (a representative image is shown in Fig. 1b).

Hybrids—cytogenetic characterization

DNA indexes for known diploid and tetraploid bahiagrass plants were recorded at 0.21 and 0.43–0.45, respectively. The test samples (hybrids) showed DNA indexes between 0.25 and 0.38. The tetraploid hybrid R26, obtained from non-transgenic OP tetraploid bahiagrass, showed a DNA index similar to the transgenic pollen donor B9 (Table 3). Root-tip chromosome counts of representative plants were confirmed as follows: R1, $2n = 30$; R6, $2n = 26$; R3, $2n = 34$ (Fig. 2); R2, R4, R5 $2n = 28$; $2n = 2x = 20$; B9 $= 40$ $2n = 4x = 40$, Table 3.

Hybrids- growth and reproductive characterization

Some hybrids (e.g., R7, R8, R9 and R10) did not persist under greenhouse conditions and hence could not be analyzed further. Leaf biomass of most of the persistent hybrids was not significantly different from that of non-transgenic diploid or tetraploid bahiagrass (Fig. 3a, b).

Table 1 Pollen-mediated gene transfer frequency from herbicide resistant, apomictic tetraploid bahiagrass to diploid bahiagrass, following open pollination at 0.5–1.5 m distance between pollen donor and receptor plants

Pollen receptor	No. of transgenic inflorescence	No. of plants screened	No. of hybrids
Population of Pensacola genotypes	48	8300	13 (0.16%)
Single genotype of Pensacola clonally propagated (Clone 1)	46	332	12 (3.6%)
Single genotype of Pensacola clonally propagated (Clone 2)	32	5209	0 (<0.02%)

Table 2 Pollen-mediated gene transfer frequency from herbicide-resistant apomictic tetraploid bahiagrass to tetraploid bahiagrass, following open pollination at 0.5–1.5 m distance between pollen donor and receptor plants

Pollen receptor	No. of transgenic inflorescence	No. of seedlings screened	No. of herbicide-resistant seedlings
Highly apomictic genotypes	30	6,297	10 (0.16%)
Argentine	30	3,669	4
Tifton 7	30	1,069	1
Paraguay 22	30	1,559	5
Facultative apomictic genotype	32	724	15 (2.07%)

Table 3 DNA ploidy determined through flow cytometer and karyotypic observations of hybrids obtained from OP non-transgenic diploid bahiagrass

Sample	DNA index[a]	Chromosome counting (2*n*)
B9[c]	0.45	40
4*x* Known	0.43	40
2*x* Known	0.21	20
R1	0.32	30
R2	0.29	28
R3	0.34	34
R4	0.29	28
R5	0.27	28
R6	0.28	26
R8	0.38	–
R12	0.29	–
R14	0.29	–
R17	0.35	–
R18	0.36	–
R19	0.37	–
R20	0.38	–
R21	0.28	–
R26[b]	0.45	–
R27	0.38	–

– Chromosome counting was not attempted in this sample

[a] DNA index is the ratio of sample/internal standard DNA fluorescence; chicken erythrocytes nuclei (CEN) were used as internal standard

[b] R26 is hybrid derived from OP non-transgenic tetraploid bahiagrass

[c] B9 is the transgenic tetraploid pollen parent

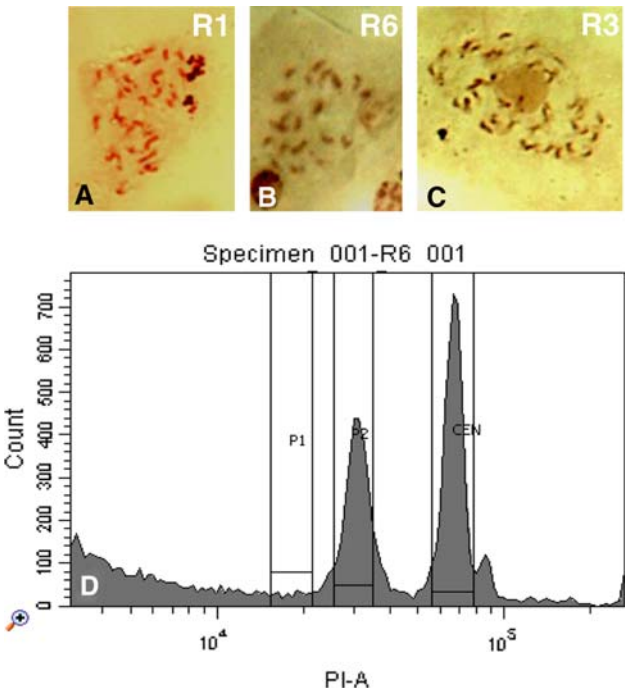

Fig. 2 Ploidy determination of F_1 hybrids with chromosome counting and flow cytometry. Root-tip chromosome preparations of F_1 hybrids showing **a** R1 with $2n = 30$, **b** R6 with $2n = 26$ and **c** R3 with $2n = 34$. Roots fixed with 95% ethanol: acetic acid, 3:1 v/v were squashed using 45% acetic acid and stained with aceto-orcein. Chromosomes were observed under 500×–1,000× magnification. **d** A flow cytometer histogram of hybrid R6 showing peak P2, representing the sample, and peak CEN for the internal standard

Hybrids R2, R5 and R20 produced the lowest biomass of all persistent hybrids, which was significantly lower than the non-transgenic tetraploid, but not significantly lower than the non-transgenic diploid. On the other hand, hybrids producing the highest biomass, including R1, R21 and R27, produced the highest biomass of all persistent hybrids, which was significantly more than the non-transgenic

diploid, but not significantly higher than the non-transgenic tetraploid (Fig. 3b). Seed head production also showed variability. No hybrid produced significantly more seed heads than the non-transgenic tetraploid, while hybrids R8, R17 and R20 produced significantly fewer seed heads than the non-transgenic Argentine bahiagrass (Fig. 3c). Hybrid R2 did not produce seed heads and also displayed a distinctive dwarf phenotype with higher tiller number and fine leaves. Seed set (also referred to as seed production) of all hybrids was significantly lower than that of the non-transgenic tetraploid (Fig. 3d). Hybrid R26, obtained from OP tetraploid cultivar (Paraguay 22), produced the highest leaf biomass and seed set as compared to other hybrids from OP diploids. However, like other hybrids from OP diploids, R26 did not exceed the non-transgenic tetraploid bahiagrass in either leaf biomass or seed set (Fig. 3).

Embryo sacs of five hybrids verified as near triploids, which also flowered under natural photoperiod (R1, R3, R4, R5 and R6) were characterized. A majority of the ovules in hybrids R1, R3, R5 and R6 showed multiple pairs of central cells and an organized meiotic embryo sac (Table 4; Fig. 4a). A single meiotic embryo sac was occasionally found in inverted orientation in some ovules of hybrids R1, R3, R4 and R5 (Table 4). The percentage of aborted ovules was as high as 50% in hybrid R3. Pollen viability in hybrids R1, R3, R4, R5 and R6 varied between 60 and 75% (Fig. 4b, c; Table 4).

When diploid pollen receptors were used, there was segregation of the apospory locus in the herbicide-resistant hybrids. The apospory-specific RFLP marker, with a hybridization signal of 4.9 kb, was detected in 13 out of 15 hybrids analyzed (Fig. 5a), suggesting that hybrids R7 and R17 reproduce sexually. These hybrids did not produce any viable seeds. When tetraploid cultivars were used as pollen receptors, the apospory-specific RFLP marker, with a hybridization signal of 4.9 kb, was detected in all 12 herbicide-resistant tetraploid hybrids analyzed (Fig. 5b).

Discussion

The existence of transgenic hybrids, resulting from transgene escape from genetically modified (GM) crops to wild relatives, is well documented, but the fate of the transgenes over time in populations of recipient species is still relatively unknown (Warwick et al. 2008). This is the first report of intra-specific pollen-mediated transgene flow from apomictic tetraploid bahiagrass to diploid or tetraploid bahiagrass, including vegetative, reproductive and cytogenetic characterization of resulting hybrids.

Risk assessment of transgenic crop species has to be addressed on a case by case basis, due to the complex nature of gene flow and transgene introgression (Chapman and Burke 2006). The frequency of gene flow depends on: (a) the existence of synchronous flowering between the transgenic and the non-transgenic counterpart, (b) compatibility between genotypes or the mating system, and (c) mode of pollination. Introgression depends on the hybridization rate, the fitness of hybrids, and the behavior/effect of the introgressed gene(s). Among other factors affecting gene flow, the particular characteristics of the habitat where a species is grown is an important criteria, especially for crop species grown in areas other than its center of diversity (Messeguer 2003).

We detected an average 0.16% gene transfer frequency from transgenic tetraploid apomictic bahiagrass to a synchronously flowering population of sexual diploid bahiagrass genotypes or apomictic tetraploid cultivars, at 0.5–1.5 m distance. The observed gene transfer rate is low compared to reports from sexual cross-pollinating (Cunliffe et al. 2004; Watrud et al. 2004; Wang et al. 2004; Bae et al. 2008; Busi et al. 2008) and facultative apomictic grasses (Johnson et al. 2006). However, our study was conducted under controlled greenhouse conditions and a field study would be desirable to confirm the observed gene transfer rate. In contrast to field studies, greenhouse experiments facilitate synchronization of flowering of different genotypes, which should enhance the expression of amphimixis (Espinoza et al. 2002). A low frequency of pollen-mediated gene transfer (0.09–0.32%) between apomictic tetraploid cultivars was detected by the herbicide resistance marker. Earlier studies predicted the potential for sexual or apomictic seed production in bahiagrass with embryos sac analysis (Quarin and Hanna 1980; Quarin et al. 1982; Acuna et al. 2007) or with the help of the observed transgene integration and segregation patterns (Sandhu and Altpeter 2008). The use of self-incompatible clonal diploids as pollen receptors, and transgenic tetraploids as pollen donors, demonstrated that pollen competition is a significant factor affecting gene flow. This approach is similar to the use of male-sterile receptor plants designed to reduce pollen competition (Wang et al. 2006; Hoyle et al. 2007; Jia et al. 2007; Yuan et al. 2007).

The present greenhouse study confirmed that pollen from tetraploid transgenic apomictic bahiagrass can produce viable seeds on sexual diploid bahiagrass pollen receptor plants, given that there is synchronous flowering under natural conditions. A theoretical assessment has shown that even low hybridization rates of 0.1% could lead to the establishment of a moderately advantageous ($s = 0.10$) transgene (Haygood et al. 2004). Hence, besides evaluating short and long range hybridization frequencies under field conditions, it is important to study the fate of such hybrids (Chapman and Burke 2006; Hails and Morley 2005). In the present study, we have primarily focused on the fate of hybrids formed on sexual diploid pollen

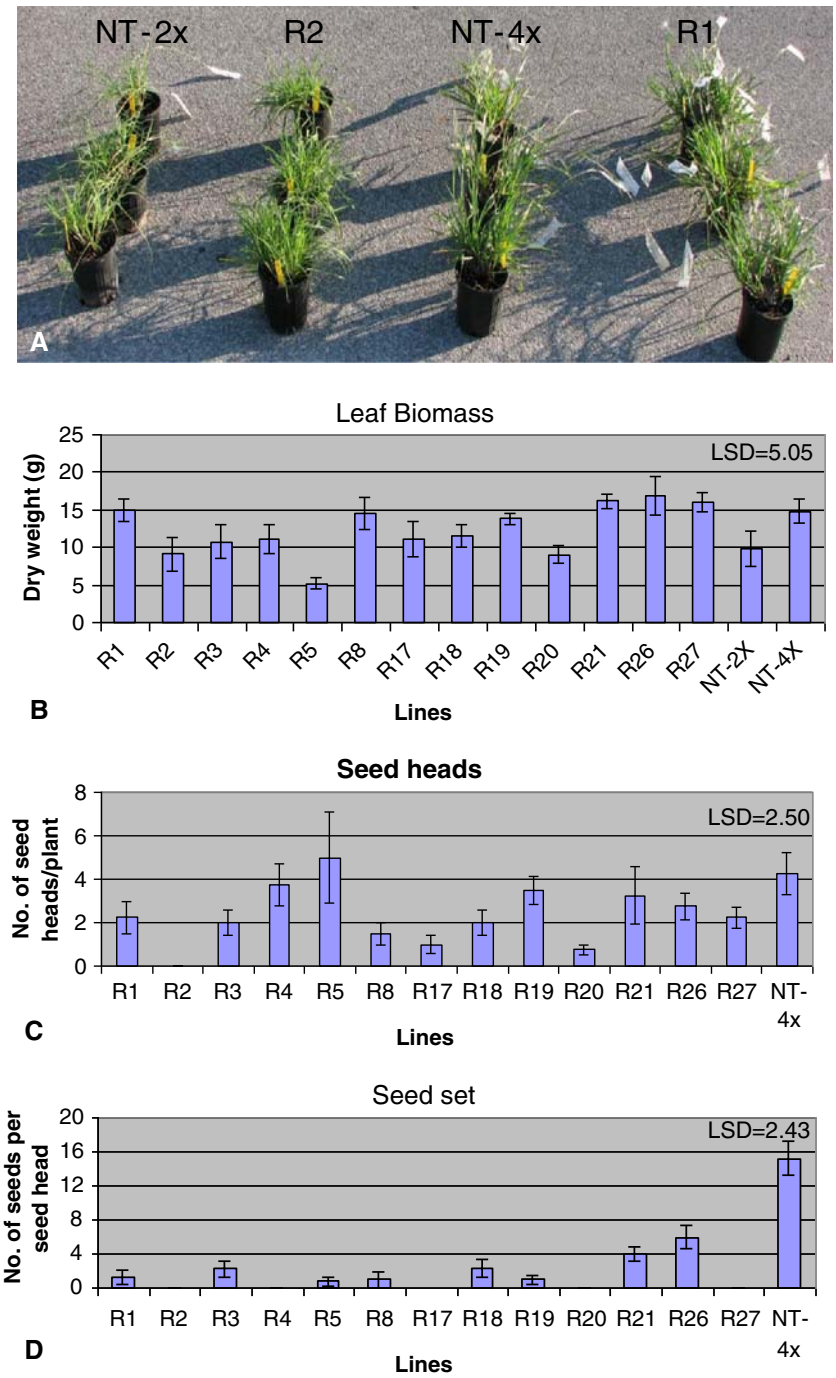

Fig. 3 Biomass and seed set in F_1 hybrids. **a** A representative picture of hybrids R1 and R2, non-transgenic diploid (NT-2*x*), and non-transgenic tetraploid (NT-4*x*) bahiagrass. **b** Leaf biomass of hybrids in comparison with NT-4*x* and NT-2*x* bahiagrass. **c**, **d** Seed head and seed set of hybrids in comparison to NT-4*x* bahiagrass. Hybrids R1, R2, R3, R4, R5, R8, R17, R18, R19, R20, R21 and R27 were aneuploids originating from OP sexual diploids; hybrid R26 originated from OP highly apomictic tetraploid bahiagrass

receptors. Fate of hybrids is determined by their fitness characteristics such as survival, fertility, mode of reproduction and reproductive compatibility (Chapman and Burke 2006; Jackson et al. 2004). Hybrid fitness is the best predictor of allelic spread (Chapman and Burke 2006).

In *Paspalum limbatum*, a spontaneous hypotriploid hybrid, originating from a diploid plant and an apomictic tetraploid male parent, was characterized as apomictic but seed sterile (Acuna et al. 2004). Spontaneous or manual production of triploids in different *Paspalum* species has resulted in male sterile hybrid plants. Acuna et al. (2004) reported that the hypotriploid hybrid was 100% male sterile. Hanna and Burton (1986) reported 0.02% viable pollen from a *P. notatum* apomictic triploid, originating from a cross between a facultative apomictic tetraploid female and a sexual diploid male parent. Several (near) triploid hybrids produced in this study, from crosses of a sexual diploid as the female and an apomictic tetraploid as the male parent, produced viable pollen, although seed set was limited. Hanna and Burton (1986) reported a greater

Table 4 Pollen viability and embryo sac types in hybrids obtained from OP sexual diploid bahiagrass

Hybrid	Pollen viability (%)	No. of ovules studied	No. of ovules with			
			Apomictic	Apomictic + sexual	Sexual	Aborted
R1	70	37	0	21	7[a]	9
R3	75	40	0	17	3[b]	20
R4	60	33	12	8	3[a]	10
R5	60	33	4	20	5[a]	4
R6	60	30	0	15	2	13

[a] One ovule had single sexual embryo sac with inverted orientation

[b] Two ovules had a single sexual embryo sac with inverted orientation

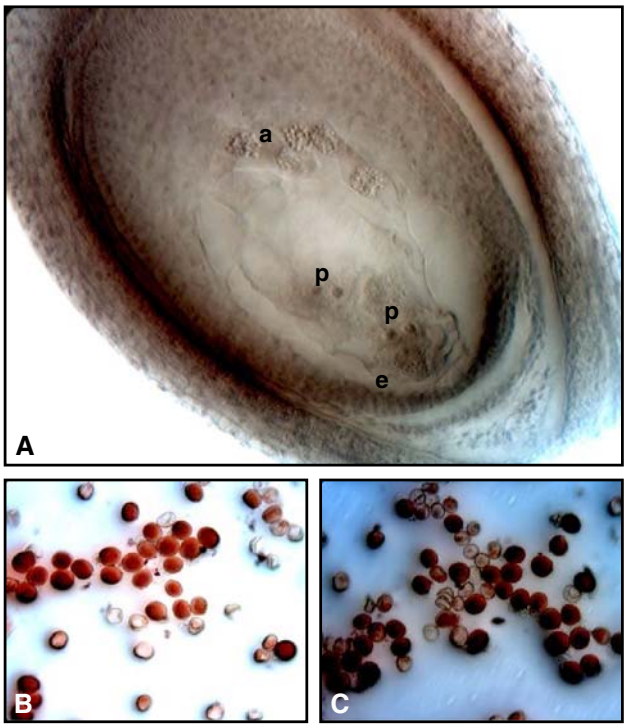

Fig. 4 Reproductive characterization of F_1 hybrids. **a** An ovule bearing multiple embryo sacs from hybrid R6. *a* antipodals, *e* egg cell, *p* polar nuclei. **b**, **c** Stained pollen collected from hybrid R6. *Dark red* staining indicates viable pollen, while *light* or *transparent* pollen are non-viable

vigor of their triploid hybrid plant compared to tetraploid progenies. However, we found that most (near) triploid or tetraploid hybrids did not differ in biomass production compared to both non-transgenic diploid and tetraploid bahiagrass, although seed set was significantly lower than the non-transgenic tetraploid bahiagrass. Acuna (2006) reported an average seed set of 39% for OP diploids and 36% for OP tetraploids. Interestingly, hybrid R5 showed the lowest biomass, highest seed head production and yet only 3.8% seed germination.

Flow cytometry and karyotypic observations showed irregular chromosome numbers in most of the hybrids obtained from a sexual diploid female and tetraploid apomictic pollen donor. Triploid progeny of sexual diploid female and apomictic tetraploid male plants have irregular chromosome numbers in *Taraxacum* sect. *Ruderalia* (Martonfiova et al. 2007). Chromosome elimination during microsporogenesis has been reported in *P. subciliatum* (Adamowski et al. 1998). Chromosome elimination has been widely observed in inter-specific crosses or in allo-tetraploid genomes (Gernand et al. 2006; Adamowski et al. 1998). The tetraploid accessions of *P. notatum* originated from autopolyploidization and are known to show high frequencies of meiotic irregularities (such as anaphase laggards, micronuclei formation), and abnormal tetrads leading to unbalanced gametes (Quarin et al. 1984; Quarin 1992; Adamowski et al. 2005). Chromosome number variability observed in the aneu-triploid hybrids could be generated by such meiotic instabilities during microspore formation in tetraploid pollen donors, and may cause reduced fitness.

The RFLP C1069 marker is considered a simple dose restriction fragment (Martinez et al. 2003) and is expected to segregate 1:1 for presence:absence in the F_1 generation for a simplex (A—) condition (Wu et al. 1992). Earlier reports have suggested 3:1 expression of sexuality/apomixis in segregating tetraploid hybrid populations (Stein et al. 2004; Martinez et al. 2001). Such distortion towards sexuality has been explained by a lethal dominant effect of the apospory gene. Apospory in *Bracharia* spp., *Panicum maximum* and *Pennisetum ciliare* had been confirmed as a single dominant gene with tetrasomic inheritance (Do Valle et al. 1994; Savidan 1981; Sherwood et al. 1994). Stein et al. (2004) suggested disomic inheritance based on AFLP markers linked to the apo-allele, and preferential chromosome pairing in the apospory-specific genomic region (ASGR). In the present study open-pollination of sexual diploid female plants with transgenic apomictic tetraploid male plants resulted in 13 apomictic and 2 sexual F_1 hybrids, based on RFLP analysis. Cytogenetic observations of the embryo sac of five hybrids showed sexual or facultative apomixis in at least four of the five hybrids.

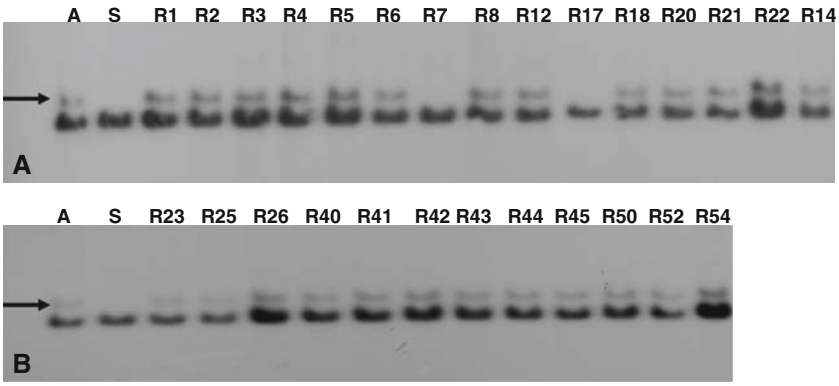

Fig. 5 Molecular marker analysis of F_1 hybrids using a RFLP marker linked to apospory in tetraploid *P. notatum*. **a** RFLP analysis of F_1 hybrids obtained from sexual diploid pollen receptors open-pollinated with transgenic apomictic tetraploid bahiagrass as the pollen donor. **b** RFLP analysis of F_1 hybrids obtained from apomictic tetraploid pollen receptors open-pollinated with transgenic apomictic tetraploid bahiagrass as the pollen donor. Genomic DNA digested with *Eco*RI was hybridized with the C1069 rice probe. *Arrows* indicate the apospory-specific 4,900 bp restriction fragment. *A* tetraploid aposporous, *S* diploid sexual, *R* herbicide resistant F_1 hybrids, *R1-R7* hybrids originating from a population of sexual diploid genotypes, *R8-R22* hybrids originating from a clonal diploid genotype, *R23-R45* hybrids originating from highly apomictic tetraploid genotypes and, *R50-R54* hybrids originating from facultative apomictic tetraploid genotypes

Interestingly, all herbicide-resistant tetraploid hybrids carried the apospory-specific RFLP marker. Linkage of the *bar* gene to the aposporus locus is an unlikely cause for this result, since segregation of the aposporus RFLP marker was detected in herbicide-resistant (near) triploid hybrids derived from the same pollen donor. Therefore, a low degree of amphimixis in the apomictic bahiagrass genotypes used as the pollen receptor is the only plausible explanation for a low frequency of pollen-mediated gene transfer between apomictic tetraploid genotypes.

Thus, tetraploid apomictic bahiagrass does not provide complete transgene containment under unfavorable conditions. However, intra-specific gene transfer from apomictic bahiagrass is drastically reduced compared to sexually reproducing perennial grasses, and the majority of resulting hybrids produced limited seeds. The presented data also demonstrates that apomictic Argentine bahiagrass is an excellent source of genetic variability that can be released when the apomictic male is crossed with a sexual female. A wide range of variability was generated by $2x \times 4x$ (transgenic) hybridization, ranging from non-persistent hybrids, non-flowering dwarf-type hybrids (R2) with good vigor, aneuploids with reduced seed head production, and triploids that exhibit the vigor and seed head production of non-transgenic bahiagrass, but with significantly reduced seed set (R1). Non-flowering aneu-triploids represent the highest level of transgene containment. Such hybrids with good vigor/field performance and dwarf phenotype (such as hybrid R2) may be desirable for turf applications, which would justify vegetative propagation for establishment of new plantings.

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References

Acuna CA (2006) Bahiagrass germplasm reproductive characterization, and breeding at the tetraploid level. M.S. thesis, University of Florida, Gainesville

Acuna CA, Martinez EJ, Quarin CL (2004) Apospory followed by sterility in a hypotriploid hybrid ($2x \times 4x$) of *Paspalum*. Caryologia 57:373–378

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

Adamowski E, Pagliarini MS, Batista LAR (1998) Chromosome elimination in *Paspalum subciliatum* (Notata group). Sex Plant Reprod 11:272–276

Adamowski E, Pagliarini MS, Bonato ABM, Batista LAR, Valls JBM (2005) Chromosome numbers and meiotic behavior of some *Paspalum* accessions. Genet Mol Biol 28:773–780

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

Altpeter F, James VA (2005) Genetic transformation of turf-type bahiagrass (*Paspalum notatum* Flugge) 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

Busi R, Yu Q, Barrett-Lennard R, Powles S (2008) Long distance pollen-mediated flow of herbicide resistance genes in *Lolium rigidum*. Theor Appl Genet 117:1281–1290

Caceres ME, Matzk F, Busti A, Pupilli F, Arcioni S (2001) Apomixis and sexuality in *Paspalum simplex*: characterization of the mode of reproduction in segregating progenies by different methods. Sex Plant Reprod 14:201–206

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

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5:213–218

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 Agr Res 55:389–396

Daurelio LD, Espinoza F, Quarin CL, Pessino SC (2004) Genetic diversity in sexual diploid and apomictic tetraploid populations of *Paspalum notatum* situated in sympatry and allopatry. Plant Syst Evol 244:189–199

Dixon L, Nyffenegger T, Delley G, Martinezizquierdo G, Hohn T (1986) Evidence for replicative recombination in Cauliflower Mosaic-Virus. Virology 150:463–468

Do Valle CB, Glienke C, Leguizamon GOC (1994) Inheritance of apomixis in Brachiaria, a tropical forage grass. Apomixis Newsl 7:42–43

Dolezel J, Lysak MA, Houwe IV, Dolezelova M, Roux N (1997) Use of flow cytometry for rapid ploidy determination in *Musa* species. Infomusa 6:6–9

Dolezel J, Greilhuber J, Suda J (2007) Estimation of nuclear DNA content in plants using flow cytometry. Nat Protoc 2:2233–2244

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

Espinoza F, Daurelio LD, Pessino SC, Vall EM, Quarin CL (2006) Genetic characterization of *Paspalum notatum* accessions by AFLP markers. Plant Syst Evol 258:147–159

Gates RN, Quarin CL, Pedreira CGS (2004) Bahiagrass. In: Warm-season (C4) grasses, Agronomy monograph no 45s

Gernand D, Rutten T, Pickering R, Houben A (2006) Elimination of chromosomes in *Hordeum vulgare* × *H.* bulbosum crosses at mitosis and interphase involves micronucleus formation and progressive heterochromatinization. Cytogenet Genome Res 114:169–174

Gondo T, Tsuruta S, Akashi R, Kawamura O, Hoffmann F (2005) Green, herbicide-resistant plants by particle inflow gun-mediated gene transfer to diploid bahiagrass (*Paspalum notatum*). J Plant Physiol 162:1367–1375

Hails RS, Morley K (2005) Genes invading new populations: a risk assessment perspective. Trends Ecol Evol 20:245–252

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

Haygood R, Ives AR, Andow DA (2004) Population genetics of transgene containment. Ecol Lett 7:213–220

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

Jackson MW, Stinchcombe JR, Korves TM, Schmitt J (2004) Costs and benefits of cold tolerance in transgenic *Arabidopsis thaliana*. Mol Ecol 13:3609–3615

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* Flugge) enhances abiotic stress tolerance. Transgenic Res 17:93–104

Jia S, Wang F, Shi L, Yuan Q, Liu W, Liao Y, Li S, Jin W, Peng H (2007) Transgene flow to hybrid rice and its male-sterile lines. Transgenic Res 16:491–501

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

Kron P, Suda J, Husband BC (2007) Applications of flow cytometry to evolutionary and population biology. Annu Rev Ecol Evol Syst 38:847–876

Luciani G, Altpeter F, Yactayo-Chang J, Zhang H, Gallo M, Meagher R, Wofford D (2007) Expression of *cry1Fa* in bahiagrass enhances resistance to fall armyworm. Crop Sci 47:2430–2436

Martinez EJ, Urbani MH, Quarin CL, Ortiz JPA (2001) Inheritance of apospory in bahiagrass, *Paspalum notatum*. Hereditas 135:19–25

Martinez 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

Martinez EJ, Acuna CA, Hojsgaard DH, Tcach MA, Quarin CL (2007) Segregation for sexual seed production in *Paspalum* as directed by male gametes of apomictic triploid plants. Ann Bot 100:1239–1247

Martonfiova L, Majesky L, Martonfi P (2007) Polyploid progeny from crosses between diploid sexuals and tetraploid apomictic pollen donors in *Taraxacum* sect. Ruderalia. Acta Biol Crac Ser Bot 49:47–54

Matzk F, Meister A, Schubert I (2000) An efficient screen for reproductive pathways using mature seeds of monocots and dicots. Plant J 21:97–108

Messeguer J (2003) Gene flow assessment in transgenic plants. Plant Cell Tissue Org 73:201–212

Mogie M (1988) A model for the evolution and control of generative apomixis. Biol J Linn Soc 35:127153

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

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

Nogler GA (1984) Genetics of apospory in apomictic *Ranunculus auricomus* V. Conclusions. Bot Helv 94:411–422

Ochatt S (2008) Flow cytometry in plant breeding. Cytometry 73A:581–598

Ortiz JPA, Pessiono SC, Leblanc O, Hayward MD, Quarin CL (1997) Genetic fingerprinting for determining the mode of reproduction in *Paspalum notatum*, a subtropical apomictic forage grass. Theor Appl Genet 95:850–856

Ozias-Akins P (2006) Apomixis: developmental characteristics and genetics. Crit Rev Plant Sci 25:199–214

Pupilli F, Lambobarda P, Caceres ME, Quarin CL, Arcioni S (2001) The chromosome segment related to apomixis in *Paspalum simplex* is homeologous to the telomeric region of the long arm of rice chromosome 12. Mol Breed 8:53–61

Quarin CL (1992) The nature of apomixis and its origin in panicoid grasses. Apomixis Newsl 5:8–15

Quarin CL, Hanna WW (1980) Effect of three ploidy levels on meiosis and mode of reproduction in *Paspalum hexastachyum*. Crop Sci 20:69–75

Quarin CL, Hanna WW, Fernandes A (1982) Genetic studies in diploid and tetraploid *Paspalum* species—embryo sac development, chromosome behavior and fertility in *Paspalum cromyorrhizon, Paspalum laxum and Paspalum proliferum*. J Hered 73:254–256

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

Quarin CL, Norrmann GA, Urbani M (1989) Polyploidization in aposporous *Paspalum* species. Apomixis Newsl 1:28–29

Quarin CL, Espinoza F, Martinez 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

Roux N (2003) Rapid detection of aneuploidy in *Musa* using flow cytometry. Plant Cell Rep 21:483–490

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* Flugge). 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

Savidan Y (1981) Genetics and utilization of apomixis for the improvement of guineagrass (*Panicum maximum* Jacq.). Proc XIV Intl Grassland Congr, Lexington, pp 182–184

Sherwood RT, Berg CC, Young BA (1994) Inheritance of apospory in buffelgrass. Crop Sci 34:1490–1494

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

Stein J, Quarin CL, Martinez 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

Stewart CN Jr, Halfhill MD, Warwick SI (2003) Transgene introgression from genetically modified crops to their wild relatives. Nat Genet 4:806–817

Taliaferro CM, Hopkins AA, Henthorn JC, Murphy CD, Edwards RM (1997) Use of flow cytometry to estimate ploidy level in *Cynodon* species. Int Turfgrass Soc Res J 8:385–392

Thompson CJ, Movva NR, Tizard R, Crameri R, Davies JE, Lauwereys M, Botterman J (1987) Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. EMBO J 6:2519–2523

Tischler CR, Burson BL (1995) Evaluating different bahiagrass cytotypes for heat tolerance and leaf epicuticular wax content. Euphytica 84:229–235

Valkonen JPT (1994) Nuclear DNA content of the *Solanum* spp. In the series etuberosa as determined by laser flow cytometry. Ann Appl Biol 125:589–600

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 J, Yang X, Li Y, Elliot PF (2006) Pollination competition effects on gene flow estimation: using regular vs. male-sterile bait plants. Agron J 98:1060–1064

Warwick SI, Legere A, Simard M-J, James T (2008) Do escaped transgenes persist in nature? The case of an herbicide resistance transgene in a weedy Brassica rapa population. Mol Ecol 17:1387–1395

Watrud LS, Lee HE, Fairbrother A, Burdick C, Reichman JR, Bollman M, Strom M, King Gm, Van de Water PK (2004) Evidence of landscape-level, pollen-mediated gene flow from genetically modified creeping bentgrass with CP4 EPSPS as a marker. Proc Natl Acad Sci USA 101:14533–14538

Wu KK, Burnquist W, Sorells ME, Tew TL, Moore PH, Tanksley SD (1992) The detection and estimation of linkage in polyploids using single-dose restriction fragments. Theor Appl Genet 83:294–300

Young BA, Sherwood RT, Bashaw EC (1979) Cleared-pistil and thick-sectioning techniques for detecting aposporous apomixis in grasses. Can J Bot 57:1668–1672

Yuan QH, Shi L, Wang F, Cao B, Qian Q, Lei XM, Liao YL, Liu WG, Cheng L, Jia SR (2007) Investigation of rice transgene flow in compass sectors by using male sterile line as a pollen detector. Theor Appl Genet 115:549–560

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