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Comparative gene expression in sexual and apomictic ovaries of *Pennisetum ciliare* (L.) Link.

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Abstract

Limited emphasis has been given to the molecular study of apomixis, an asexual method of reproduction where seeds are produced without fertilization. Most buffelgrass (*Pennisetum ciliare* (L.) Link syn = *Cenchrus ciliaris* L.) genotypes reproduce by obligate apomixis (apospory); however, rare sexual plants have been recovered. A modified differential display procedure was used to compare gene expression in unpollinated ovaries containing ovules with either sexual or apomictic female gametophytes. The modification incorporated end-labeled poly(A)$^+$ anchored primers as the only isotopic source, and was a reliable and consistent approach for detecting differentially displayed transcripts. Using 20 different decamers and two anchor primers, 2268 cDNA fragments between 200 and 600 bp were displayed. From these, eight reproducible differentially displayed cDNAs were identified and cloned. Based on northern analysis, one cDNA was detected in only the sexual ovaries, two cDNAs in only apomictic ovaries and one cDNA was present in both types of ovaries. Three fragments could not be detected and one fragment was detected in ovaries, stems, and leaves. Comparison of gene expression during sexual and apomictic development in buffelgrass represents a new model system and a strategy for investigating female reproductive development in the angiosperms.

Introduction

In many angiosperms sexuality is associated with or replaced by apomixis, an asexual method of reproduction that culminates in the formation of viable embryos genetically identical to the maternal plant [3, 4, 5, 7, 13]. The genetic transfer of apomixis to sexual crops could revolutionize current breeding strategies for seed production by allowing the generation of self-perpetuating improved hybrids, and the fixation of desired genotypes. Genetic studies have demonstrated that the most prevalent forms of apomixis are genetically inherited and are controlled by a few genes [16].

While several apomictic mechanisms have been described in the plant kingdom [2], apospory is prevalent in the Poaceae. Apospory is a form of apomixis in which somatic cells in the ovule give rise to unreduced ($2n$) female gametophytes [9]. The autonomous development of aposporous egg cells generates viable embryos without fertilization; however, the majority of aposporous species are pseudogamous and require fertilization of the polar nuclei for endosperm development [16]. Most aposporous plants are facultative apomicts where sexual and apomictic development coexist within the ovule or the inflorescence. The formation of sexual and aposporous female gametophytes occurs simultaneously, and depends on distinct developmental pathways that overlap in time and space. The reproductive fate (sexual or apomictic) can be influenced by environmental factors [11, 12], making a

The nucleotide sequences reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers U65082 (Pcs-1), U65383 (Pcs-2), U65384 (Pcs-3), U65385 (Pcs-4), U65386 (Pca-1), U65387 (Pca-2), U65388 (Pca-3), U65389 (Pca-4).

molecular comparison of sexuality and apomixis particularly difficult in facultative apomicts.

Buffelgrass (*Pennisetum ciliare* (L.) Link syn = *Cenchrus ciliaris* L.) is a warm season perennial forage grass widely cultivated throughout the semi-arid tropics. In contrast to other apomictic grasses, most buffelgrass genotypes reproduce by obligate apospory [18]. In buffelgrass, aposporous unreduced ($2n$) female gametophytes can be cytologically identified because they lack antipodal cells [21]. Rare obligately sexual plants that only form reduced ($1n$) female gametophytes of the Polygonum type (two synergids, an egg cell, two polar nuclei in the central cell, and a cluster of antipodals) have been recovered. Hybridization of sexual and aposporous genotypes has resulted in hybrid populations (F_1) which contain plants that segregate for method of reproduction [19]. The presence of obligately apomictic and obligately sexual F_1 buffelgrass genotypes provide unique germplasm to investigate sexuality and apomixis at the molecular level.

The spatial and temporal patterns of megaspore mother cell (MMC) differentiation and meiosis are the same during sexual and aposporous development in buffelgrass; however, in aposporous ovules, one or several nucellar cells are able to pursue growth and differentiation. In obligately apomictic plants, all meiotically derived megaspores abort and reduced female gametophytes are not formed. After cellularization, four-nucleated female gametophytes differentiate into an egg apparatus and a central cell containing usually one or two polar nuclei. Aposporous female gametophytes exhibit rapid degeneration of the synergids and precocious development of zygote-equivalent egg cells that can give rise to organized proembryos in unpollinated pistils [20].

In this study a new strategy for the simultaneous identification of genes differentially expressed during either sexual or apomictic reproduction is presented. The approach is based on a modified differential display procedure that allows the comparison of mRNA populations present at specific stages of ovary development. The analysis was conducted using sexual and obligately aposporous genotypes selected from an F_1 population segregating for method of reproduction. An external morphological parameter (ovary diameter) was used to identify equivalent stages of female gametophyte development in sexual and aposporous pistils. Differential display was used to identify and characterize cDNA sequences corresponding to transcripts present within the ovary during the development of either sexual or aposporous female gametophytes.

Materials and methods

Plant material

Three obligately apomictic genotypes (BBW220, BBW266, and BBW291) and one obligately sexual genotype (BBW178), from an F_1 population of buffelgrass were selected and grown in a greenhouse (T° = 15–30 °C; RH = 60–90%). Their method of reproduction and female gametophyte development of the selected genotypes was determined using a clearing procedure [21]. Ovary diameter was estimated under bright field and phase contrast observations using a stage micrometer. Sectioned material was processed as previously described [20].

RNA isolation and cDNA synthesis

Mature unpollinated pistils with ovaries ranging from 0.3 to 0.5 mm in diameter were dissected from the florets of a sexual (BBW178) and an apomictic (BBW266) genotype and placed in liquid nitrogen. After removing the stigmas and styles from the ovaries, total RNA was extracted from fresh frozen ovaries (ca. 250 ovaries per extraction) using the procedure described by Chomczynski and Sacchi [6]. Duplicated batches of first strand cDNA were synthesized using single 5′-T_{11}XY-3′ anchor primers in a 25 μl reaction mixture containing: 1 or 2 μg of total RNA, 2.5 μM of a single anchor primer, 20 μM of each dNTP, 500 μM DTT, and 250 units of MMLV reverse transcriptase (Superscript, Gibco-BRL[1]). Reaction tubes containing all reagents were incubated at 37 °C for 1 hour. cDNA reactions were aliquoted and kept frozen at −80 °C.

Differential display

Individual ^{32}P end-labeled T_{11}-XY oligonucleotides were synthesized in 50 μl reactions containing: 950 μM of the specific T_{11}VN, 2 μg of bovine serum albumin (BSA), 5 μl (125 pmol) of ^{32}P-γ-ATP (6000 Ci/mmol), and 20 units of T4 polynucleotide kinase (NEB[1], Beverly, MA). After incubation at 37 °C for 1 h, the primer was ethanol precipitated at −20 °C for 12 h, washed in 95% ethanol, and resuspended in 50 μl of water.

[1]Mention of a trade mark of proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of the products that may also be suitable.

Polymerase chain reaction (PCR) was performed in mixtures containing 2 μl of the reverse transcription reaction, 5 μl of the appropriate ^{32}P-labeled $T_{11}VN$, 1 × PCR reaction buffer, 20 μM of each dNTP, 0.5 μM specific arbitrary decamer oligonucleotide (Operon) and 10 units of AmpliTaq DNA[1] Polymerase (Perkin Elmer Cetus[1], Norwalk, CT). Mineral oil (Sigma[1]) was overlaid and PCR was performed at 94 °C for 30 s, 35 °C for 1 min, 72 °C for 30 s, during 40 cycles, followed by 5 min at 72 °C and a 4 °C hold. Each PCR was performed twice with two different cDNA batches corresponding to 1 and 2 μg total RNA in the initial cDNA synthesis. To ensure that total RNA samples were largely DNA-free, PCR controls in which the reverse transcriptase was omitted were performed during initial experimental stages. Sequencing stop buffer (USB[1], Cleveland, OH) was added to each PCR microfuge tube. Samples were pre-heated at 75 °C for 2 min. and electrophoresed in 6% polyacrylamide sequencing gels for 4 to 5 h at 60 W constant power in 1 × TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH = 8). After vacuum drying, gels were exposed to Kodak[1] XAR-5 Film for 16 to 48 h, depending on their specific activity.

DNA fragments larger than 200 bp in length and showing reproducible differential signals with titrated cDNA samples were excised by carefully aligning the gel to the autoradiogram. To verify appropriate band recovery, gels were re-exposed to film. Excised slices were rehydrated in TE buffer (10 mM Tris, 1 mM EDTA, pH = 8) and DNA was eluted by boiling the samples for 15 min. After ethanol precipitation with 50 μg glycogen as a carrier, DNA was immediately re-amplified by PCR using appropriate primers and the same conditions as those described above. Products of reamplification were visualized on a 1.5% agarose gel and stored at −20 °C. When differentially displayed fragments were excised from the polyacrylamide gel and re-amplified using PCR, some exhibited several bands of different molecular size on agarose gels. To identify the band that was differentially displayed in the autoradiogram, PCR was conducted with the same cDNA template but different combinations of flanking primers. In most cases, the fragment of interest could only be amplified if both the oligo(dT) and the arbitrary decamer were included in the PCR.

Cloning and sequencing

Reamplified cDNA products (see above) were cloned into the pCRII vector using the instructions of the TA Cloning kit (Invitrogen[1], San Diego, Cal.), or by electroporation of DH10B (Gibco-BRL[1]) competent cells. DNA sequencing was performed directly from the vector using the Taq Dye Primer Cycle Sequencing Kit and analyzed using the 373 DNA Sequencing System (Applied Biosystems[1], Perkin-Elmer). Sequence data base searches were performed using programs based on the BLAST algorithm [1]. The BLASTX and BLASTN programs were used to screen the nucleotide and amino acid sequence version of the data bases held at the National Center for Biotechnology Information, National Institute of Health, Bethesda, MD.

RNA gel blot hybridizations

Total RNA was extracted from leaves, stems and ovaries according to the procedure previously described [6]. For each type of tissue, isolation of mRNA from 100 μg total RNA was conducted using the PolyATract mRNA Isolation System (Promega[1]). Isolated samples of mRNA were denatured with formaldehyde, electrophoresed in 1% agarose-formaldehyde gels, blotted onto Hybond N^+ membranes (Amersham[1]), and hybridized with random-primed ^{32}P-labeled cDNA. After hybridization, blots were washed in 2 × SSC, 0.1% SDS for 15 min at 23 °C, and twice in 1 × SSC, 0.1% SDS for 15 min at 65 °C. Hybridization signals were detected by exposing the membranes to Kodak XAR-5 film, or using a Phosporimaging System (Fujix[1] BAS2000). Blots were probed several times after removing the hybridizing probe cDNA by treatment with 0.5% SDS at 100 °C for 10 min.

Results

Modifications to differential display

Differential display of mRNA offers the opportunity to compare gene expression patterns between tissues where sample size is limiting, and where total RNA has to be used as a template for cDNA synthesis. To assess the overall performance of differential display, preliminary experiments were performed using total RNA extracted from aerial tissues of *Arabidopsis thaliana* (cv. Landsberg). Using the conventional differential display protocol [14], all amplified cDNA fragments that were cloned and sequenced were flanked only by the arbitrary decamer, and not by the $T_{11}VN$ (where V = A, G or C and N = A, T, G, or C) poly(A)$^+$ primer.

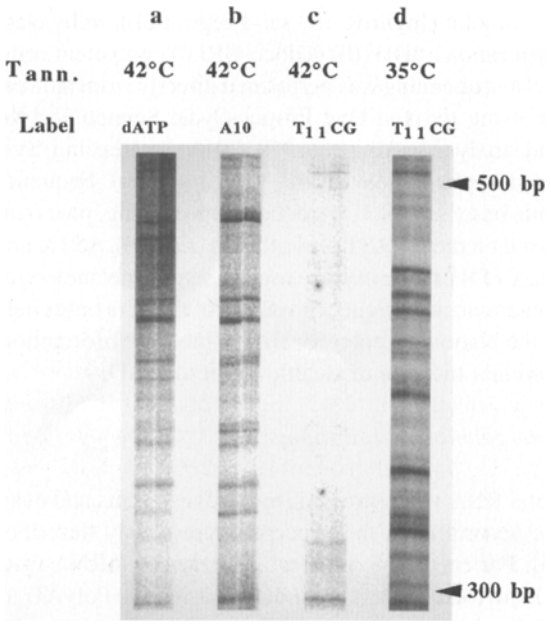

Figure 1. Comparison of different labeling procedures on differential display band detection. Differential display was performed on independent cDNA samples (lanes 1 and 2) prepared from 1 μg of total RNA extracted from *Arabidopsis thaliana* (cv. Landsberg) leaf tissue. These cDNA templates were then used for differential display under the following conditions; **(a)** ^{32}P-dATP was added to the PCR and the annealing temperature was 42 °C; **(b)** the arbitrary decamer A10 (5′-GTGATCGCAG-3′) primer was end-labeled with ^{32}P and used to detect amplified cDNAs in a PCR reaction with a 42 °C annealing temperature; **(c)** the T_{11}CG primer was end-labeled with ^{32}P and used to detect amplified cDNAs in a PCR reaction with 42 °C as the annealing temperature; **(d)** the T_{11}CG primer was end-labeled with ^{32}P and used to detect amplified cDNAs in a PCR reaction with 35 °C as the annealing temperature.

To determine the priming efficiency of each oligonucleotide, differential display was performed using the same 1st strand cDNA template, but PCR amplified cDNAs were radioactively labeled by adding either an isotopically labeled T_{11}VN oligonucleotide, a labeled arbitrary decamer, or ^{32}P-dATP to the reaction mix. The results of these experiments are presented in Fig. 1. When conventional differential display was used at an annealing temperature of 42 °C, consistent bands were observed if ^{32}P-dATP was used in the PCR reaction (Fig. 1a). These bands corresponded to amplified cDNA fragments labeled with the arbitrary decamer (Fig. 1b). Only a few of these bands corresponded to cDNA fragments labeled with the T_{11}VN oligonucleotide (Fig. 1c). Similar results were obtained with different primer combinations (data not shown), suggesting that the priming efficiency of the T_{11}VN primer is low under conventional differential display PCR conditions. Based on these results, end-labeled T_{11}VN oligonucleotides were used as the only isotopic source in subsequent differential display reactions, and PCR conditions were modified to improve priming efficiency. The optimal annealing temperature was determined to be 35 °C (Fig. 1d). We subsequently determined that increasing the concentration of each deoxyribonucleotide (dNTP) to 20 μM increased the signal of each amplified cDNA product. These modifications increased the reliability of differential display, as a reproducibility greater than 90% was obtained in independent PCR experiments.

Ovary growth and female gametophyte development

To identify comparable stages in ovaries of genotypes selected for gene expression comparison, the development of the female gametophyte was correlated with external morphological parameters. While style length was found to be highly variable, ovary diameter appeared to follow reproductive development within the ovule of the genotypes studied. On the basis of this analysis, three ranges of ovary diameter were identified that correspond to overlapping stages of female gametophyte formation. In the sexual genotype (BBW178), ovaries having a diameter of less than 0.2 mm contained pre-meiotic megaspore mother cells (MMCs), MMCs undergoing meiosis, or two nucleated female gametophytes. In the aposporous genotype (BBW266), ovaries with a diameter less than 0.2 mm contained ovules undergoing megasporogenesis, at the early stages of nucellar induction, or containing 2-nucleated embryo sacs. Sexual ovaries ranging from 0.2 to 0.35 mm in diameter contained only non-cellularized embryo sacs. In contrast, cellularized aposporous female gametophytes were found in ovaries ranging from 0.2 to 0.5 mm in diameter. In sexual ovaries, cellularized female gametophytes were found only in ovaries having a diameter greater than 0.35 mm.

Differential gene expression in sexual and aposporous ovaries

Differential display was used to compare gene expression during sexual and aposporous development in buffelgrass ovaries. Developmental stages corresponding to cellularized female gametophytes were compared by extracting total RNA from ovaries with a diameter greater than 0.35 mm. These results are illustrated in Fig. 2. A single combination of primers gener-

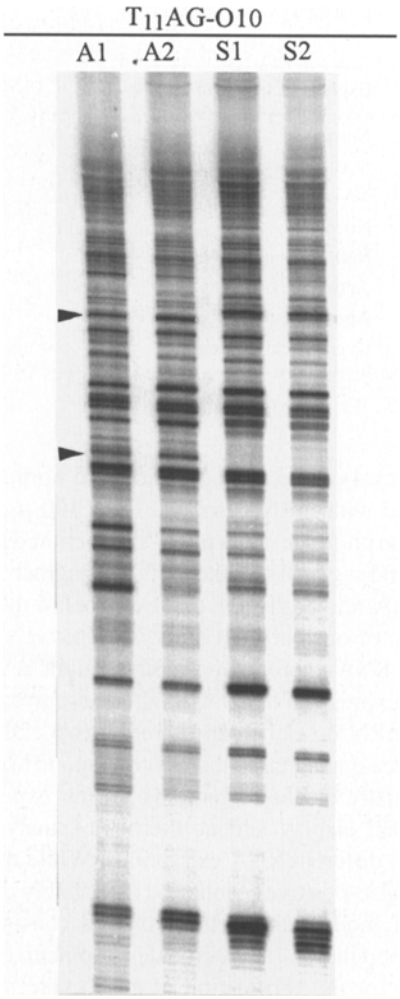

Figure 2. Comparison of mRNA subpopulations in ovaries of buffelgrass. Differential display from ovaries of an aposporous (lanes A1 and A2) and a sexual (lanes S1 and S2) genotype of *P. ciliare*. A selected poly(A)$^+$-anchored primer (T_{11}AG) was used to synthesize first-strand cDNA using either 1 μg (lanes A1 and S1) or 2 μg (lanes A2 and S2) of total RNA as a template. The same primer was then end-labeled with ^{32}P and combined to an arbitrary primer (3′-TCAGAGCGCC-5′) in a PCR reaction. Amplification products were separated in a 6% polyacrylamide gel. Arrowheads indicate the presence of bands present in aposporous but not sexual ovaries.

ated 50 to 60 amplified bands with sizes ranging from 200 to 600 bp. PCR was performed with two different cDNA samples corresponding to titrated amounts of total RNA (1 μg and 2 μg total RNA, respectively). About 95% of displayed cDNA fragments in that range could be reproduced with titrated cDNA samples. A majority of the fragments corresponded to mRNAs present in both sexual and aposporous ovaries.

To identify genes differentially expressed in sexual and aposporous ovaries, specific anchor primers were combined with 20 different decamers in independent PCR reactions. These combinations generated 2268 fragments from which eight reproducible differentially displayed fragments between 200 and 600 bp in size were recovered. The primer combinations and fragment sizes are presented in Table 1. DNA sequences were used to determine that differentially displayed fragments were flanked by the correct decamer at their 5′ end, and the poly(A)$^+$ tail-specific anchor primer at the 3′ end. Comparison to nucleotide or amino acid sequences contained in the data bases of the National Center for Biotechnology Information (NIH, Bethesda, MD) revealed a similarity between Pcs-2 and a transcript expressed in vegetative meristems of maize (*Zea mays* cDNA clone 7C03B09). The seven other fragments did not show a homology with sequences contained in these databases.

To verify the differential expression of genes corresponding to differentially displayed fragments, cDNA clones were used as probes in RNA gel blot analysis. Blots employing 20 μg of total RNA revealed that one cDNA fragment (Pca-4) was expressed in leaves, stems, and ovaries. The differential expression of the remaining fragments could not be confirmed, as no signal was detected under different stringency conditions.

The specific expression of four clones (Pcs-2, Pca-1, Pca-2 and Pca-3) was confirmed using poly(A)$^+$ mRNA-enriched blots from 100 μg total RNA. These results are summarized in Table 1 and Fig. 3. One fragment (Pca-1) was detected at similar levels only in ovaries of sexual and apomictic plants, but not in vegetative tissues. Three cDNA fragments (Pcs-2, Pca-2, and Pca-3) corresponded to transcripts specifically expressed either in sexual or aposporous ovaries. The remaining four differentially displayed fragments (Pcs-1, Pcs-3, and Pcs-4) could not detect mRNA signals in poly(A)$^+$ RNA blots.

Discussion

Molecular studies of gene expression during apomictic development have not been reported. Reasons for the lack of research are that the female gametophyte is less accessible than the microgametophyte and only a few cells per ovule are involved in sexual or apomictic development. In sexual species, gene expression studies have required laborious efforts of dissection and enzymatic maceration to isolate developing female

Table 1. Differentially displayed cDNA fragments isolated from mature ovaries of buffelgrass.

Name	10-mer	Poly(A)$^+$	Size	Expression in RNA blots
Pcs-1	5′-GTAGCACTCC-3′	5′T_{11}GC3′	416	No signal[a]
Pcs-2	3′-CCACGGGAAG-5′	5′T_{11}GC3′	287	Sexual ovaries[a]
Pcs-3	3′-CAGCACTGAC-5′	5′T_{11}AG3′	287	No signal
Pcs-4	3′-GACAGGAGGT-5′	5′T_{11}AG3′	377	No signal
Pca-1	3′-CAGCACTGAC-5′	5′T_{11}AG3′	253	Both types of ovaries[a]
Pca-2	3′-TCAGAGCGCC-5′	5′T_{11}AG3′	340	Apomictic ovaries[a]
Pca-3	3′-CTGTTGCTAC-5′	5′T_{11}AG3′	274	Apomictic ovaries[a]
Pca-4	3′-CCACGGGAAG-5′	5′T_{11}AG3′	210	Ovaries, leaves, stems[a]

[a] Expression analysis of these clones is presented in Fig. 3.

gametophytes from their surrounding sporophytic tissue [8]. Differential display of mRNA does not require the construction of cDNA libraries, and allows gene expression analysis using microgram quantities of total RNA for first-strand cDNA synthesis [14]. Differential display has been successfully used to study gene expression in plants [17], and has resulted in the isolation of genes involved in embryo development [10].

The small size of the buffelgrass ovary limits the amount of mRNA that can be isolated and used for molecular studies. To overcome this limitation, differences in gene expression between sexual and apomictic ovaries were analyzed with differential display. An initial assessment revealed that the performance of the technique is dependent on the labeling procedure used to detect amplified cDNA fragments. Based on comparisons of different labeling procedures, our data suggest that the use of an isotopically labeled T_{11}VN primer in the PCR is the most reliable strategy to identify and clone mRNA-related cDNA fragments when total RNA is used as the initial template. This approach insures that PCR products contain the poly(A)$^+$ T_{11}VN primer on at least one end, and avoids detection of fragments that are only flanked by the arbitrary decamer. Additionally, a lower annealing temperature in the PCR improves the priming efficiency of the T_{11}VN oligonucleotide. The high reproducibility obtained with titrated cDNA samples suggests that the detection of rare transcripts differentially expressed in the ovary is difficult, but can be accomplished [15]. In this study, the expression of 30% of the differentially displayed cDNAs was confirmed by northern blot analysis.

Three cDNAs (Pcs-2, Pca-2 and Pca-3) corresponding to transcripts specifically expressed in ovaries obtained from aposporous or sexual plants were identified. Three differentially displayed fragments (Pcs-1, Pcs-3, Pcs-4) could not be detected in northern blots generated with mRNA isolated from 100 μg of total RNA. The presence of the poly(A)$^+$ anchored primer at their 3′ end suggests that these cDNA fragments represent mRNA-related cDNAs expressed below the level of detection of our northern blots. Alternative strategies such as RNAse protection assays might reveal patterns of expression of cDNA fragments corresponding to rare mRNAs. Gene expression of two cDNA fragments (Pca-1 and Pca-4) did not correspond to expected differentially displayed patterns. In our experiments, differential display and northern blot analysis were based on different RNA extractions. While the detection of false-positives cannot be completely discarded with our modifications, the temporal consistency of mRNA populations at specific developmental stages is required for the verification of gene expression. The presence of undifferentiated female gametophytes in ovaries with a diameter greater than 0.35 mm may also have an influence in gene expression patterns. Comparative gene expression in advanced stages of ovary growth may identify differentially displayed cDNAs corresponding to genes expressed during undifferentiated stages of megagametogenesis; such genes may be ectopically expressed in sexual or apomictic ovaries.

Gene expression comparisons were conducted in a subset (ca. 20%) of mRNA species present in ovaries containing cellularized female gametophytes. Based on the analysis of 2268 displayed fragments, most genes follow the same patterns of expression in mature ovaries of full-sib sexual and obligately aposporous buffelgrass. Limited differences detected in gene expression may be related to female gametophyte development during either sexual or apomictic reproduction. Under this assumption, molecular comparisons of mature ovaries in buffelgrass could result in

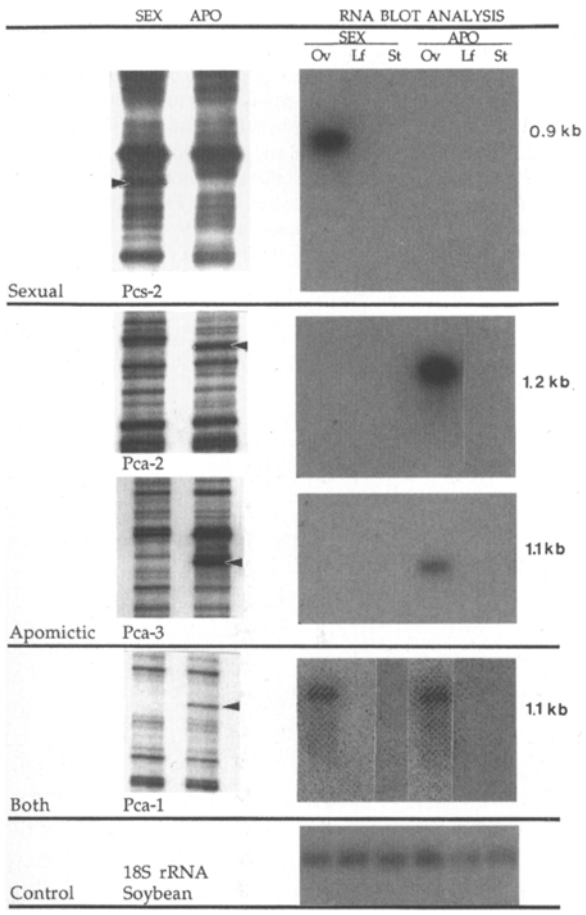

Figure 3. Differential gene expression in ovaries of buffelgrass. Four differentially displayed bands (arrowheads) in sexual (SEX) or aposporous (APO) ovaries were isolated and cloned in a PCRII™ vector. RNA gel blots were prepared from poly(A)$^+$-enriched RNA generated with 100 μg of total RNA extracted from ovaries (Ov) leaves (Lf) and stems (St) from a sexual (SEX) and an aposporous (APO) genotype. Blots were probed with the corresponding cDNA clones isolated from differential display and signal was detected using X-ray film (12 to 14 days exposure; Pcs-2, Pca-2, and Pca-3) or with a phosphorimaging system (Pca-1). The approximate length of each transcript is indicated at the far right. Reprobing with the soybean 18S rRNA unit allowed the quantity of RNA in each lane to be compared.

the identification of genes involved in autonomous egg cell development (parthenogenesis), gamete fusion, or antipodal cell differentiation.

This study shows that buffelgrass can be used as a model system for molecular comparisons of sexual and apomictic reproduction. Differential display is a valid and reproducible procedure to compare gene expression in sexual and apomictic female organs. Comparative studies may reveal new insights on functional aspects of female gametophyte development, and on developmental mechanisms leading to the evolution of reproductive strategies in flowering plants.

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