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REVISTA DE INVESTIGACIONES DE LA FACULTAD DE CIENCIAS AGRARIAS - UNR

V Ciclo de Seminarios dobre avances en la Caracterización Genética y Molecular de la APOMIXIS

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Durante el periodo comprendido entre el 14 y el 16 de Noviembre de 2016 se realizó el V Ciclo de Seminarios sobre Avances en la Caracterización Genética y Molecular de la Apomixis, organizado por el Departamento de Agronomía de la Universidad Nacional del Sur y el CERZOS-CONICET, Bahía Blanca. Esta reunión tuvo como objetivo continuar con el ciclo de encuentros bienales que, desde el año 2008, se realizan alternativamente en el Departamento de Agronomía de la Universidad Nacional de Sur o en la Facultad de Ciencias Agrarias de la Universidad Nacional de Rosario. El evento está enfocado en discutir los resultados de los grupos argentinos que investigan el fenómeno de apomixis (reproducción asexual vía semillas), de manera que sus integrantes adquieran una visión global del tema bajo estudio y tengan la oportunidad de desarrollar cooperaciones. Estos encuentros constituyen una excelente oportunidad para la formación de los estudiantes de doctorado que trabajan en la temática. A ellos asisten profesores extranjeros, quienes amplían el contexto desde el cual se analizan los datos al aportar los resultados de sus propias investigaciones. Asimismo, sus visitas crean oportunidades para establecer o fortalecer colaboraciones internacionales.

La apomixis es una forma de reproducción muy particular, que involucra la formación de semillas en ausencia de meiosis y fecundación. Estas semillas contienen embriones clonales, genéticamente idénticos a la planta madre. Se considera que el carácter surgió repetidas veces durante la evolución, como consecuencia de alteraciones de los patrones clásicos de la reproducción sexual por causas genéticas y epigenéticas. La apomixis tiene la propiedad de fijar las estructuras genéticas heterocigotas de los genotipos híbridos por un número indefinido de generaciones, por lo que puede ser utilizada de manera programada en los planes de mejoramiento vegetal, acelerándolos y reduciendo su costo de manera dramática. Varias especies forrajeras apomícticas naturales de los géneros *Brachiaria* y *Paspalum* están siendo mejoradas en planes que utilizan esta capacidad de reproducción clonal vía semillas. El desarrollo de estos programas está posibilitando una mejora significativa en la capacidad de producción de forrajes para las áreas subtropicales de Sudamérica. Sin embargo, la mayoría de los grandes cultivos se reproducen por sexualidad y carecen de parientes apomícticos reproductivamente compatibles. Para ampliar el objetivo de asistir al mejoramiento a esos cultivos mayores se requiere un conocimiento exhaustivo de las bases moleculares que controlan el fenómeno. En los últimos años se han producido grandes avances en este sentido, impulsados por los proyectos de secuenciación de ARN pequeños, transcriptomas, y genomas, que están revolucionando la capacidad de generación de herramientas biotecnológicas útiles.

A este V Ciclo de Seminarios sobre Avances en la Caracterización Genética y Molecular de la Apomixis asistieron investigadores y becarios de las Universidades Nacionales del Sur y de Rosario. Los profesores extranjeros invitados fueron el Dr. Olivier Leblanc, del Institut de Recherche pour le Développement, Montpellier, Francia; el Dr. Emidio Albertini de Department of Agricultural, Food and Environmental Sciences, University of Perugia, Italia; y el Dr. John Carman, del Plants, Soils and Climate Department, Utah State University, Logan, UT, USA. Se presentaron un total de 15 trabajos, cuyos resúmenes se publican a continuación.

Apomixis in eukaryotes: an ancient fair-weather alternative to sex

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Resumen

A characteristic of eukaryotes is the production of discrete, epigenetically-reset cells that re initiate the life cycle, a process that generally occurs in association with meiosis and syngamy. However, it also occurs in eukaryotes by the formation of discrete, epigenetically-reset gametes or gamete-like cells that form without meiosis and re-initiate the life cycle without syngamy. This is called apomixis, and it occurs with sex in all kingdoms of eukaryotes, e.g., in single-celled protists and fungi, in primitive multicellular plants and animals, and in more derived plants and animals. The most primitive apomicts today are single-celled haplontic protists, and among them are those where apomixis cycles with sex (in an organism) in response to environmental cues. Apomixis is the fair-weather mode. Sex occurs in response to metabolic stress. Similar environment-regulated sex-apomixis switches are found in organisms from all five eukaryote kingdoms. Consequently, we have hypothesized that apomixis is an epigenetically-regulated life-cycle alternative that originated with single-celled haplontic protozoa during eukaryogenesis where it cycled with sex in response to the environment. If correct, two key implications emerge. First, apomeiosis is not just unreduced gamete formation. It is "formation, without chromosome reduction, of parthenogenesis-competent gametes or gamete-like cells." This definition links all types of apomeiosis together by a conserved, epigenetics-based apomixis-life-cycle mandate, including no meiosis, as occurs in primitive haplontic apomicts, and derived forms of apomeiosis where meiosis is avoided or modified, as occurs in apospory and diplospory, apogamy and adventitious embryony, and automictic parthenogenesis. Secondly, the hypothesis implies an absence of *de novo* apomixis genes, the implication being that altered levels of expression of normal genes cause epigenetic shifts toward an apomictic or a sexual fate.

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Array-based comparative study of transcripts expressed in flowers of apomictic and sexual genotypes of *Eragrostis curvula*

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Resumen

Apomixis (asexual seed production) is characterized by meiotically unreduced egg cell production (apomeiosis) followed by parthenogenetic development into offspring that are genetic clones of the mother plant. The aim of this work was to identify transcripts that are differentially expressed between apomictic and sexual genotypes of Eragrostis curvula by using the one-color (Cy3), 60-mer oligonucleotides microarray platform of Agilent. Previously, a floral reference transcriptome for apomictic and sexual genotypes of this grass was constructed using total RNA from flowers at different developmental stages from the tetraploid genotypes Tanganyika USDA (apomictic) and OTA USDA (sexual). Two samples from each genotype were collected (two different plants, biological replicas constituted by a mix of different developmental stages) and sequenced by using the 454 GS FLX+ Roche method, according to the protocol provided by the manufacturer. Transcripts de novo assembly (Newbler software package) gave a total of 49,568 contigs (~80,000,000 bp) and 133,782 singletons (~40,000,000 bp). This information and previous sequenced ESTs of this grass were used to design a 1-million spotted chip array (Agilent) using the software provided by the manufacturer. Hybridizations were preformed using RNA samples from four diplosporous tetraploid genotypes (Tanganyika, Don Walter, Ermelo and Morpa) and from four sexual genotypes (OTA (4x) and the diploids (2x) Victoria and accessions PI299920, PI208214). Resulting raw data from the Agilent's Feature Extraction (AFE) software were pre-processed and analyzed with GeneSpring GX, a multi-omics data analysis software. Preprocessing consisted on a percentile shift of 75% normalization, using the mean values of reproductive mode, apomictic or sexual, as parameters. Subsequent analysis of the normalized data identified 110 transcripts differentially expressed (p-values <0.01) between the apomictic and sexual genotypes. Some of the genes that were repressed in the apomictic group compared with the sexual one belong to the histone superfamily, 40S ribosomal transcripts and rRNA methyltransferases. The up-regulated genes (fold change >2 and p values >0.01) in the apomictic genotypes compared with the sexual ones were mainly hypothetical proteins, some of them with the conserved domain MIF4G or belonging to the E3 ubiquitin ligase complex. Several cyclin isoforms, transcription factors, F-box with WD repeats and leucine-rich repeats, B-box zinc finger, LRR receptor-like serine/threonine-protein kinase and Dna J domains associated with hsp70 heat-shock system were all up-regulated in the apomictic genotypes. The most important result of this work is the finding of three transcripts that are significantly more differentially expressed between apomictic and sexual genotypes. These transcripts correspond to an unknown sequence that is exclusively present in apomictic genotypes, a cyclin and a splicing factor. We are actually working in order to establish the relationship of the expression of these genes and apomixis in Eragrostis curvula.

Deciphering apomixis-specific regions using *Paspalum*

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Resumen

Apomixis in flowering plants covers a wide range of reproductive behaviours that lead to seeds harbouring maternal embryos. Apomictic species likely evolved from sexual ancestors through alterations in the regulation of the core developmental programs governing sexual reproduction. They usually form agamic complexes; i. e. sexuality and apomixis are found in different cytological compartments, with the sexual plants usually restricted to the lowest ploidy level, while apomicts are polyploid. The developmental alterations underlying apomixis are genetically determined, but the nature of both the loci and the mechanisms remains under debate. Genetic studies in several grasses (Pennisetum squamulatum, Tripsacum dactyloides, Brachiaria decumbens, Paspalum notatum) have revealed that apomixis is under the control of a limited number of loci, usually mapped in a chromosomal region affected by suppression of recombination and biased transmission (so called Apomixis-Specific Region or ASR). Further cytogenetic and sequence characterization in Pennisetum and Paspalum have shown that the ASR contains both intact and truncated genic sequences, interspaced by large retrotransposon-rich sequences. Interestingly, in contrast to the complexity found in polyploid Paspalum notatum individuals, evidences in other Paspalum species indicate contrasted situations for the ASR organization and behaviour, including: expression of apomixis at the diploid level; lower or no suppression of recombination, and absence of transmission bias. These variations question the functional role of ASR structural organization in the transition from sexuality to apomixis. To understand both the ASR organization and its role in installing apomixis in plants, we recently initiated genome sequencing of a diploid Paspalum notatum genotype that shows some levels of apomixis expression. By using the most recent sequencing technologies in genotypes covering ASR variations, we will further produce genomic and epigenomic data, to identify both regulatory and genic sequences responsible for the ASR functional role. Moreover, we will explore the evolutionary forces that shaped its (epi)genomic patterns. With regard to this, evolutionary scenarios will also be discussed.

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Looking for candidate genes for apomixis

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Resumen

In the first part of this talk will be focused on *Poa pratensis*, where we have isolated a gene, termed *APOSTART* (Albertini et al. 2005, Plant Phys 138:2185-2199). Our previous results demonstrate that some *APOSTART* members are expressed exclusively in inflorescences and, overall, our results suggest that *APOSTART* may be related to the programmed cell death that is involved in the non-functional megaspore and nucellar cell degeneration events that permit enlargement of maturing embryo sacs. In addition, to put to the test the hypothesis of an involvement of *APOSTART* in apomixis, we have characterized *APOSTART* members from two other aposporic species. Our results, showing that at least one *APOSTART* member/allele is expressed differentially in all species, shed light on the possible role of *APOSTART* in apomixis. To better understand this function, we are characterizing *Arabidopsis thaliana APOSTART* members. In particular, *PpAPO1* shares high homology with the *Arabidopsis* gene AT5G45560, thus renamed *AtAPOSTART1* (*AtAPO1*), and with with *EDR2* (*ENHANCED DISEASE RESISTANCE 2*). Tang and co-workers proved that *EDR2* disruption enhances *Arabidopsis* capacities to resist to *E. cichoracearum* infections (Tang et al. 2005; Vorwerk et al., 2010); *edr2* homozygous plants does not show any developmental defects and are indistinguishable from the wild type ones, whilst *ATAPO1* down-regulation affects negatively seed germination. In order to verify if *ATAPO1* and *EDR2* have additive or redundant roles we generated and analyzed the *atapo1-2*/*edr2* double mutants. They appear smaller than the two parental lines, and, interestingly, the developing siliques are smaller. Manual dissection of the siliques showed that also seed development is compromised. Microscopic analyses show that all seeds contain embryos, 30% of which show a delayed or arrested development. Moreover, by using a computational approach we have determined the tri-dimensional structure of the START domain, with the aim of investigating the possible interactions between the START domain and one or more phytosterols (i.e. stigmasterol, campesterol, brassicasterol) which are believed to be important during the embryogenesis. The second part of the talk will be focused on candidate genes for apomixis which could be epigenetically regulated. In fact, some studies have demonstrated that during sexual reproduction a genome-wide decrease of DNA methylation takes place and is compensated by de novo methylation. In the present study, we investigated DNA methylation in pre-meiotic flower buds of four diploid sexual species: *Boechera retrofracta* (Utah); *Boechera stricta* (Utah); eastern (Cody, Wyoming) and western (Imnaha, Oregon) sexual genotypes of *Boechera microphylla*; and four diploid apomictic *Boechera* species or species hybrids: *Boechera lignifera* (Wyoming); *Boechera retrofracta* x *stricta* (Colorado): *Boechera retrofracta* x *exilis* (Utah) and *Boechera microphylla* (Utah). Cytological analyses revealed diplospory in the apomictic genotypes except for *Boechera microphylla*, which was aposporous. Our aim was to identify epigenetic differences in flower buds between sexual and apomictic species by analyzing DNA methylation. A total of 1203 clear and reproducible bands were amplified. The extent of DNA methylation ranged from 70.55% in apomictic *Boechera retrofracta* to 83.54% in *Boechera yellowstonensis*. In particular DNA methylation averaged 77.53% in apomictic species and 81.12% in sexual species. We then checked the entire collection for polymorphisms and found 10 that were absent in all sexual species and present in all apomictic species or vice versa. Amplicons were sequenced and the resulting genes were checked for their expression. RNA-seq data confirmed that the genes affected by differential methylation are highly differential expressed between apomictic and sexual *Boechera* species.

RNA-seq and *de novo* assembly of sexual and apomictic floral transcriptomes of *Paspalum notatum*. A key database for studying apomixis candidate genes

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Resumen

Apomixis is a natural form of asexual reproduction by seeds present in more than 35 flowering plant families. Progenies derived from apomictic plants are genetically identical to the maternal parent. This type or reproduction originates after bypass the fundamental aspects of plant sexual reproduction: meiosis and fertilization. Apomictic plants are able to form either embryos or female gametes from unreduced somatic cells. The mechanisms involved are diverse, but all of them are considered deviations from the typical sexual reproductive pathway, probably caused by genetic and/or epigenetic factors affecting one or few genes. This trait has significant value for agriculture. Transferring apomixis into economically important crops could allow the fixation of hybrid combinations, the generation of new intergeneric and interspecific hybrids adapted to local environments and the utilization of seeds in crops that are propagated by vegetative organs. *Paspalum* is one of the largest genus of the *Poaceae* family. It includes several species that are important natural forage resources for the tropical and subtropical region of South America. Over the past five decades, a wealth of information regarding the biology, genetic and reproductive behavior of many *Paspalum* species has been produced. Particularly, *P. notatum* and *P. simplex* became valuable models for the study of apomixis because they concurrently represent systems for mining candidate gene(s) and important forage crops. In both species, a number of genes associated with the trait have been identified by genetic and/or expression analyses. However, apomictic races are polyploid, highly heterozygous and have a relatively large DNA content. Therefore, the identification of fundamental genes has been difficult. Moreover, no reference genome is still available, and the number of characterized sequences deposited in databanks is still limited. In this context, global transcriptomic approaches can contribute significantly to the elucidation of the molecular bases of the trait. RNA-Seq technology offers several key advantages over other existing methodologies for characterizing transcriptomes. It is particularly attractive for non-model organisms with genomic sequences that are yet to be determined. The use of Next-Generation Sequencing (NGSs) technologies does not require neither cloning libraries nor any prior knowledge of the species genome. Particularly, 454/Roche has become a method of choice for analyzing transcriptomes of non-model organisms, because of its long-read capacity, which makes data more amenable to *de novo* assembly and annotation. The objectives of this work were: 1) to produce robust reference transcriptome datasets from flowers of sexual and apomictic genotypes, and 2) to deliver a list of transcripts with differential representation between sexual and apomictic types. A long-read 454/Roche FLX+ sequencing strategy was used for database construction. Raw data originated from sexual and apomictic flowers collected from premeiosis to anthesis was used to assembly three libraries: i) sexual, ii) apomictic and iii) global (including both reads). The global assembly produced 48,842 floral transcripts, including 67,617 allelic variants. A group of physically-supported mRNA and EST sequences was matched with high level of confidence to both sexual and apomictic libraries. Dozens of molecular functions and biological pathways operating in *P. notatum* flowers were identified. A preliminary trial allowed discovery of the whole set of putative alleles/paralogs corresponding to 23 previously identified apomixis-associated candidate genes. Moreover, a list of 3,732 genes and several co-expression networks differentially represented between both plant types were detected. The reference floral transcriptomes reported here will be of interest to plant reproductive research and *Paspalum* breeding projects.

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Effects of stress on reproductive mode in *Boechera* (Brassicaceae)

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Resumen

In many eukaryotic apomicts, stress activates an epigenetic switch from apomixis (unreduced gamete formation and parthenogenesis) to sex (reduced gamete formation and syngamy). For this to occur, a third polyphenic gender, an apomictic female, is often observed. She is well adapted to favorable conditions, is adept at producing clonal progeny, and, though genetically identical, often differs phenologically and morphologically from sexual females. Stress-activated epigenome reprogramming occurs in her progeny during their development such that sexually-functional males and females form. The genetically-identical siblings often mate to produce fertilized eggs, which can tolerate the stresses that induced their formation, e.g., starvation, cold, heat, desiccation, etc. When favorable conditions return, apomictic females form from the fertilized eggs. This cyclical-apomixis occurs in animals, fungi, protists, diatoms, and brown and green algae. In apomictic angiosperms, polyphenic genders are not generally recognized. Instead, apomixis is usually considered to be facultative, with seeds often forming sexually and apomictically on the same plant. However, in *Boechera* (Brassicaceae) and several other angiospermous genera, stress-induced switching from mostly apomeiotic to mostly meiotic ovule development occurs. Thus, angiosperms are similar to other eukaryotes in terms of i) tendencies for stress-induced polyphenic reversions from apomixis to sex and ii) physiological adaptations in apomicts that maximize fecundity thus facilitating geographic parthenogenesis. A primitive origins hypothesis for sex-apomixis polyphenisms that are more-or-less conserved among eukaryotes is supported.

Characterization of the floral transcriptome small RNA component from *Paspalum notatum* sexual and apomictic genotypes

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Resumen

Apomixis is an asexual mode of reproduction through seeds, resulting from an absent or aberrant meiosis (apomeiosis), fertilization-independent initiation of embryogenesis (parthenogenesis) and the formation of functional endosperm either autonomously or after fertilization (pseudogamy). It is conceived as a deviation from the sexual reproductive pathway, caused by failure or variation of one or a few genetic factors. The ability to produce clonal maternal progeny via seeds is of significant value to agriculture for its power to fix complex favorable hybrid genotypes. In the gramineae, apomixis is genetically controlled by one or a few loci, depending on the species, but expression modulation of some key developmental steps (like gametic fate acquisition and parthenogenesis) could involve epigenetic mechanisms. The objective of this work was to contribute to the study of the apomixis epigenetic control through a comparative analysis of the small RNA transcriptome component in florets of sexual and apomictic *P. notatum* plants. Small RNAs libraries were produced in triplicate from equitable mixes of spikelets collected at premeiosis, meiosis, postmeiosis and anthesis, as follows: total RNA was extracted with SV Total RNA isolation system (Promega). Samples were initially quantified using the Qubit 2.0 Fluorometer. RNA Integrity Number (RIN) was evaluated using Agilent Bioanalyzer 2100. The Small RNA libraries were prepared in accordance with the Illumina TruSeq Small RNA sample preparation guide. RNA 3' and RNA 5' RNA adapters were ligated to each end of sRNA molecules. Reverse transcription followed by PCR was used to create cDNA constructs. PCR was performed with two primers that anneal to the ends of the adapters, one of them containing index sequences. High quality small RNAs were extracted and purified from polyacrylamide gels for subsequent cluster generation. The resulting libraries were validated using the Agilent 2100 Bioanalyzer, to check the size, purity and concentration. For library pooling and sequencing, equal volumes of normalized libraries were combined, denatured and diluted in hybridization buffer. The library pool was combined with 5% PhiX Control to balance the overall lack of sequence diversity. The samples were then sequenced using the Illumina MiSeq in a 1x50bp single read run. The number of reads obtained ranged from 1,558,547 to 2,996,675, depending on the library. Reads quality controls were conducted with FastQC v.0.11.5 and multiQC. Adapters sequences were removed using the software Cut adapt v1.3. Bowtie v. 1.1.2 was used to align reads against reference genomes/transcriptomes. For read counting, the program Subread v 1.5.0-p1 (module feature counts) was used. Small RNAs from 18 to 26 nt were filtered and selected for comparative analysis. Length distribution within this fraction revealed peaks at 21 nt and 24 nt. Differential expression analysis was conducted with the softwares EdgeR and NOISeq. The sRNAs reads were mapped onto a *Paspalum notatum* 454/Roche floral reference transcriptome. Ninety-two (92) isotigs showed differential association with sRNAs at $p < 0.01$. Sixty-five of them (70.65%) consisted of ncRNAs. Twenty-seven (29.34%) were protein-coding sequences, out of which 10 corresponded to retrotransposon/transposon proteins. The rest of the protein-coding transcripts (18.47%) were included into the following ontological classes: protein degradation (7), signaling (1), transcription regulation (2), RNA degradation/processing (2), catabolism (2), transport (2), unknown (1). Therefore, 41% of the non-transposon protein-coding genes differentially regulated via sRNA mediated mechanisms corresponded to protein degradation pathways. Mapping onto the *Oryza sativa indica* databases revealed that 4 miRNA sequences were significantly overrepresented in apomictic libraries. Moreover, using a mirDeep2 customized approach we identified potential novel miRNA precursors among isotigs, predicted their structures through RNAfold and identified potential mature miRNA and star sequences. The following numbers of new miRNA sequences were predicted in the *P. notatum* sRNA libraries: Apo_1: 65; Apo_2: 37; Apo_3: 41; Sex_1: 43; Sex_2: 40; Sex_3: 24. In further work we will correlate the different libraries data in order to identify unique pre-miRNA predictions and check if some of these novel precursors are differentially active in apomictic and sexual plants.

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Progress toward the functional analysis of *TGS1* genes in *Arabidopsis thaliana*

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Resumen

Apomixis refers to various reproductive behaviors of angiosperms that result in asexual reproduction through seeds. Apomictic reproduction avoids both meiotic reduction and egg cell fertilization, generating offspring that are exact genetic replicas of the mother plant. The transfer of apomixis to cereals and others crops would greatly benefit agriculture by allowing the fixation of hybrid vigor and clonal multiplication by seeds of the best hybrids. However, to achieve this, a better understanding of both the cellular and molecular bases underlying apomictic development is required. Recently, our research group reported evidence for the association between the deregulation of several genes encoding RNA methyltransferases and apomictic development in tetraploid *Paspalum notatum* genotypes. One of these genes corresponds to a *TGS1* (trimethyl guanosylsynthase-1) homolog, named *TGS1*-like, which expression pattern is positively correlated with the rate of sexuality. While *TGS1* is conserved across all eukaryotes and contains a single methyltransferase domain, *TGS1*-like is a plant-specific gene, which includes an additional WW domain. The function of both TGS1 and TGS1-like remains uncharacterized in plants, but TGS1 performs a dual activity in yeast, animals and insects: 1) it trimethylates the 5' end of specific sn(o)RNAs involved in splicing, rRNA processing and telomerase function; and 2) it acts as a co-activator associated with PRIP in several transcription mediator complexes. In yeast, TGS1 is necessary for cell cycle progression, so defective mutants show delayed growth at low temperatures. In animals, the lack of TGS1 was associated with defective meiosis and an impaired hepatic function. The objective of this work was to functionally characterize two *tgs1-like* mutants in *Arabidopsis.* RT-PCR revealed preferential expression in reproductive tissues at pre-sporogenesis, sporogenesis and gametogenesis. Selfing of heterozygous lines and progeny genotyping revealed a strong segregation bias against both mutant and heterozygous classes (1hm:2ht:2wt), indicative of defective transmission of mutant alleles. Siliques of heterozygous and homozygous plants showed in average 51% and 62% of aborted seeds, respectively, suggesting gametophytic effects. Reciprocal crosses between homozygous and wild type plants revealed 37% of aborted seeds when the mutant allele is transmited through female gametes and 18% when transmitted through male gametes, suggesting that the female gametophyte was preferentially affected. In order to characterize *TGS1-like* expression patterns in *Arabidopsis* wild type plants were transformed with synthetic constructs carrying p*TGS1-like*/*TGS1*-like/GUS or GFP. *TGS1-like* is expressed in sub-terminal root tips, lateral root meristem, stomata, ovule primordia, undifferentiated gametophytes, egg cells and immature embryos. Cytoembryological analysis revealed developmental defects during megagametogenesis, endosperm and embryo development. Symmetry alterations were detected on the suspensor and the proper embryo. A delay in endosperm development was also evident. Finally, we are exploring TGS1-like function using different biochemical approaches including: complementation tests in yeast defective for TGS1 using different combinations of TGS1-like domains; immunoprecipitation of TGS1-like protein and RNA partner in p*TGS1*-like/*TGS1*-like/*GUS* transformants; and immunolocalization in reproductive tissues using a monoclonal antibody. The knowledge gained from this work will contribute to our understanding of the role of TGS1 proteins during reproduction in plants and to the harnessing of apomixis in crops.

Construction of a population for mapping apomixis in *Eragrostis curvula*

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Resumen

Apomixis is a form of asexual seed production that avoids both meiosis and fertilization. Although apomixis is genetically regulated and occurs naturally in more than 120 angiosperm genera that belong to approximately 40 families, its underpinnings at the molecular level are still unclear. Some genes or genomic regions have been associated with the trait. Weeping lovegrass (*Eragrostis curvula* [Schrad.] Nees) is a perennial grass native to Southern Africa that displays a type of apomixis termed pseudogamous diplospory. The *E. curvula* complex includes cytotypes with different ploidy levels (from 2x to 8x) that may undergo sexual reproduction, facultative apomixis, or obligate apomixis. *Eragrostis* type apomixis has some particularities: meiotic stages are absent, display two rounds of mitotic divisions, pseudogamia and an embryo/endosperm ploidy ratio 2:3. To obtain a mapping population for apomixis in weeping lovegrass we made different crosses between the female parent (full sexual OTA-S PI 574506 from USA) and the pollen donor (cultivars Tanganyika PI 234217 from USDA, Tanganyika from INTA and Don Walter from INTA). The plants used as female parents were not emasculated, and the hybrids plants were selected by employing molecular markers (RAPDs). After checking the putative hybrid plants by looking for markers originated from the male parent, it was established that the cross between OTA-S X Don Walter was the best option for obtaining a mapping population, because it rendered the highest number of hybrids (107 hybrids out of 400 evaluated plants). The hybrids were characterized as sexual or apomictic by cytoembryological analysis. The mode of reproduction was assessed taking into account the presence of meiotic processes or the number and position of nuclei during different stages of the embryo sac development. At the moment, 34 hybrid were characterized, showing an apomixis/sexual ratio of 21/13 (1.62/1). This ratio is similar to a previous one reported for *Eragrostis curvula* (1.71/1), which was determined by mapping phenotypic traits, and corresponds to a Mendelian genetic model with 2 epistatic dominant genes. We recently started with the genotypic characterization of the population, which will be completed with SNPs markers obtained using the genotyping-by-sequencing (GBS) technique.

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Is it possible to increase apospory and apomixis expressivity by hybridization and/or autopolyploidization in diploid *Paspalum rufum*?

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Resumen

Paspalum rufum Nees is a perennial grass that forms a multiploid complex composed of diploid (2n = 2x = 20) and tetraploid (2n = 4x = 40) cytotypes. The diploid form is sexual and highly self-sterile, while the tetraploid is a self-fertile pseudogamous aposporous apomict. Cytoembryological analysis revealed that some diploid *Paspalum* cytotypes presented aposporous embryo sacs (AES) together with the meiotic embryo sacs (MES) and also confirmed 5% of apomictic seeds produced in a self-pollination progeny. Subsequently, three natural populations of diploid *P. rufum* were analyzed, in order to evaluate the functionality of the apomixis component at the diploid level. This analysis allowed isolation of a single individual with capacity to complete apomixis (15% of the seed set), namely R5#49, which was classified as "diploid with residual apomixis" and another one, R6#45, which produced the whole seed set by sexuality. Both individual were able to produce AES in 5.8 and 13 % of their ovules, respectively. The objective of this work was to characterize the reproductive behavior (evaluating both apospory and parthenogenesis) of diploid *P. rufum* hybrids from a segregant F_1 population, and to analyze the influence of hybridity and autopolyploidy on reproductive behavior. An F_1 family of 39 individuals was created by crossing R6#45 x R5#49. Besides, two synthetic autotetraploids were obtained by colchicine treatment of R6#45 mature seeds. The hybrid origin of the F_1 progeny was confirmed by segregation analyses of a morphological trait and by the use of molecular markers. The ploidy level of experimental plants was determined by flow cytometry. The apospory expressivity was estimated by cytoembryological analysis of cleared ovaries at anthesis. Out of the 39 hybrids analyzed, 38 formed AES. The expressivity of the trait ranged from 0 to 36 %, with some individuals significantly exceeding the apospory expressivity values of the progenitors. Double-diploid plants had 25 and 32 % of its ovules carrying AES, respectively, a significantly higher rate comparing with that observed in R6#45. Then, we analyzed the effect of hybridity and polyploidy on other apomixis components. Diploid hybrids producing different AES proportion were evaluated on their ability to produce seeds through apomixis. Five individuals with apospory expressivity between 0 to 32% were induced to self-pollinate by using inter-specific, inter-ploidy pollen. The seeds DNA content was analyzed by flow cytometry, aimed at determining the embryo/endosperm DNA content ratio, which is typical of the reproductive mode (2:3 for seed produced from sexuality; 2:5 for seeds produced through apomixis). Experimental evidence indicated that most seeds were produced by selfing of MES. Only 2% and 0.8% of apomixis was detected in two individuals with relatively high apospory expressivity. Meanwhile, the pollination systems of autopolyploids were evaluated; they still hold self-incompatibility under selfing pollination condition, producing a small seed set. Moreover, flow cytometry analysis revealed a very low capacity to produce seeds by apomixis. Only one seed of 169 analyzed was produced by apomixis. A second seed was originated by cross pollination of an AES, generating a BIII hybrid. Our results revealed a high variability in apospory expressivity of diploid hybrids, suggesting multi-allelic and/or epigenetic control. The double-diploid reproductive characterization showed that apospory expressivity is highly hybridization and/or ploidy dependent. Contrastingly, an increase in apospory expressivity associated to hybridity or polyploidization is not sufficient to trigger parthenogenesis.

Identification of candidate genes showing epigenetic and/or genetic linkage with apomixis in *Paspalum notatum*

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Resumen

Apomixis is an asexual mode of reproduction described in numerous genera mainly belonging to the Rosaceae, Rutaceae, Compositae, Asteraceae and Poaceae families, which allows for the generation of viable clonal seeds by circumventing meiosis and fertilization. In apomictic plants, the female gamete (the egg) is formed from cells which were not derived from a meiotic process (therefore displaying the somatic chromosome number) and the embryo arises though parthenogenesis. Successful harnessing of apomixis in agriculture would not only facilitate the clonal propagation of any hybrid combination through seeds by an indefinite number of generations, but also alter the current plant breeding paradigm, by simplifying and reducing the costs of the programs aimed at improving varieties of major crops. The genus *Paspalum* is an attractive biological system for the study of this trait. Particularly in *Paspalum notatum*, apomixis is associated with a single genomic region with a distorted segregation ratio, showing a dominant behaviour. This "apospory-controlling region" (ACR) is a large non-recombinant chromosomal sector, plagged with retrotransposons and heavily methylated. Its demethylation was associated with severe depression or parthenogenesis, suggesting that the character could be under epigenetic control. Several genes with a known role in epigenetic regulation have already been implicated in the control of ovule cell-fate specification and embryo sac development.The objective of the research was to identify genes displaying differential methylation profiles in sexual and apomictic *Paspalum notatum* plants, by using a comparative approach based on the Methylation-Sensitive Amplification Polymorphisms (MSAP) technique. A total of 547 clear and reproducible bands were amplified by using twelve primer combinations. There were no statistically significant differences in the total quantity of methylated cytosines between sexual and the apomictic plants (the average percentages of CCGG methylation were 62.48% and 60.44%, respectively). Twenty-six DNA fragments showing a trend to differential methylation between apomictic and sexual plants were identified. From them, 12 were successfully excised from polyacrylamide gels, cloned, sequenced and used as queries in surveys onto sexual and apomictic *Paspalum notatum* 454 data libraries. Five sequences revealed positive matches. Three of them (PN_6.6, PN_8.5 and PN_G17, all of them demethylated in apomictic plants) corresponded to protein-coding genes, while PN_2.10 and PN_G44 were similar to retrotransposon sequences. qPCR analysis revealed quantitative overexpression in florets of apomictic plants for the three candidates. PN_6.6 encodes a DENN domain and a WD repeat-containing protein, which interacts with RAB and MAPKs and is involved in cell division and differentiation. PN_8.5 gene encodes a general regulatory factor 7, GF7, GF14 NU, GRF7, which is part of the SERK1 complex. PN_G17 encodes the FAR-RED IMPAIRED RESPONSE 1 protein. Experimental data suggested that PN_8.5 is genetically rather than epigenetically linked to apomixis. qRNAseq expression analysis conducted in laser-microdissected nucellar cells of *Paspalum simplex* revealed significant overexpression of PN_6.6 in apomictic plants just prior to the onset of aposporous initials. This work led to the identification of three interesting apomixis development candidate genes, which might represent valuable tools for future harnessing of the trait. Further studies are now been conducted in *Arabidopsis* mutants in order to examine the reproductive phenotypes and infer a possible role in reproductive development.

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Exploring the link between *TGS1*-like downregulation and the transition from sexuality to apomixis in *Paspalum notatum*

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Resumen

Apomixis (asexual reproduction via seeds) is an odd developmental process leading to the formation of a clonal offspring genetically identical to the mother plant, in absence of meiosis and fertilization. It is conceived as a deviation from the sexual reproductive route, caused by the emergence of variants of one to several genetic/epigenetic components. In prior work, we discovered that a paralog of gene *TGS1* (trimethyl guanosyl synthase-1), named *TGS1*-like, was differentially expressed in ovules of sexual and apomictic *Paspalum notatum* plants from premeiosis to anthesis. The expression of *TGS1*-like was downregulated in apomicts, and displayed a positive correlation with the sexuality rate. While *TGS1* encodes a short protein (~450 aa) with a methyltransferase domain, *TGS*1-like is longer (~540 aa) and display an extended N-terminal domain including a WW protein binding site. In yeast and animals, the TGS1 protein has a dual role: 1) promotes the completion of sn(o)RNAs biogenesis, by catalyzing the post-transcriptional conversion of 7-methylguanosine caps (m^7G) into 2,2,7-trimethylguanosine (m_3G), a process essential to mRNAs splicing, rRNA processing and telomerase activity; 2) acts as a PRIP (peroxisome proliferator-activated receptor-interacting protein)-associated coactivator in transcription mediator complexes. In eukaryotic non-plant model systems, the abolishment of *TGS1* function causes pre-rRNA processing deficiency, loss of nucleolar structural organization, and aberrant splicing of key regulators. This originates a wide range of phenotypic alterations, including cold-sensitive growth defects and meiosis failure. On the contrary, the function of *TGS1*-like was not characterized yet. Our objective was to study the possible role of *TGS1*-like in reproductive development. Based on the set of activities described in other taxa for its paralog, *TGS1*, we hypothesized that apomicts plants produce specific splice variants in flowers, which are concurrent with *TGS1*-like down-regulation. To put this hypothesis to the test, we characterized splice variants occurring in florets of sexual and apomictic *P. notatum* plants. A list of candidate genes with differential representation in 454/Roche floral transcriptome libraries was examined. We selected 120 candidates showing the lowest probability values for identical expression. Bioinformatic analyses indicated that 17 were possibly represented by different splice variants. Three transcripts (>10779, >22630 and >23387) were selected to be studied in a larger number of apomictic and sexual individuals. Intron-specific oligonucleotides were designed and used in RT-PCR and qPCR experiments to detect the unprocessed isotigs. For transcripts >10779 and >23387, alternative splicing was confirmed. Moreover, >23387 differential splicing was linked to the reproductive mode in 4 apomictic and 4 sexual plants. Isotig >23387 is the *Paspalum* ortholog to *Arabidopsis* gene *LHCA1*, encoding a protein that function as a membrane attachment module. The gene was associated with asymmetric division developmental pathways operating in stomata, trichomes and root epidermis cells; it is expressed in all these locations as well as in leaves, ovules and immature embryos. The splice variant differentially expressed in sexual and apomictic *Paspalum* plants is analogous to *Arabidopsis* LHCA1.4. In order to confirm a cause-effect link between *TGS1*-like activity and the occurrence of *LHCA*1.4 as well as other specific splice variants, we decided to investigate *P. notatum* sexual plants transformed with a *TGS1*-like antisense construction. A 733 bp specific CDR region (F_1 fragment) was cloned in antisense orientation within vector pAct1-gfbsd2 (pAct1-F_1as). Undifferentiated callus induced from mature seeds of sexual *P. notatum* were co-transformed with particles carrying pAct1-F_1as + pAct1-GFPBAR (containing reporter GFP and selector BAR), by using a Biomics gene gun. Two experiments involving 10 plates with 15 calluses each (and including selection and regeneration controls) were conducted. GFP fluorescence is now being controlled in roots using a Nikon E200 light transmission microscope. We plan to analyze *PNLHCA1* splice variants' representation levels by performing qPCR experiments in transformant and control plants. Moreover, we will examine alterations in reproductive development by using DIC microscopy. Similar explorations are being conducted in *Arabidopsis tgs1*-like defective mutants.

Megagasporogenesis and megagametogenesis in diploid and tetraploid cytotypes of *Paspalum rufum*

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Resumen

Paspalum rufum Nees is a robust erect perennial native grass belonging to the Poaceae family and the Panicoideae subfamily. Natural populations form a multiploid complex composed mainly of diploid (2n = 2x = 20) and tetraploid (2n = 4x = 40) cytotypes. Diploid forms are sexual and highly self-sterile, while tetraploid ones are pseudogamous aposporous apomictic and self-fertile. Previous works revealed that some diploid cytotypes of *P. rufum* are able to develop aposporous embryo sacs. Some individuals are even capable to produce up to 15% of their progenies by apomixis in interspecific and interploidy crosses. A detailed developmental calendar of tetraploid *Paspalum notatum* was previously constructed, establishing a temporal relationship between pollen and ovule development. However there is no specific information about *P. rufum* reproductive development up to date. The objectives of the present work were: 1) to characterize the reproductive structures of diploid cytotypes of *P. rufum*, in order to use them as a diploid model for apomixis research; 2) to compare the reproductive development between diploid sexual and tetraploid apomictic cytotypes. Developmental characterization was done by qualitative observations and quantitative determinations of cleared ovaries and also of sectioned stained ovaries. All diploid accessions analyzed were sexual, but produced aposporous embryo sacs in low proportions. The cytoembryological analysis allowed us to recognize, at the diploid level, all the reproductive structures of megasporocytes and megagametophytes, and to establish a useful correlation with pollen development. Both microsporocyte and megasporocyte development are perfectly coupled in the diploid cytotype at all analyzed stages. However, in tetraploid cytotypes, cytoembryological observation detected chronological differences, since megasporogenesis is delayed with respect to microsporogenesis. In accordance with these observations, meiosis also seems to be delayed in tetraploid respect to diploid cytotype, regarding integuments' growth. The analysis of aposporous development in diploids shows that aposporous initials (AI) appear while meiosis is occurring. Contrastingly, in tetraploids AIs arise when the megaspore mother cells (MMCs) differentiate. Besides, aposporous embryo sac development in the tetraploid cytotypes is advanced in relation to sexual embryo sacs development. Our results contributed enough information to construct reproductive calendars of *P. rufum* diploid and tetraploid cytotypes and also to support the existence of differences in the reproductive developmental timing between diploid and tetraploid genotypes. According to our results, the female meiosis is delayed in tetraploid genotypes.

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Exploring a possible role of floral ANK-TPR proteins in the apomixis genetic control

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Resumen

Apomixis is a natural asexual mode of reproduction by seeds, which generates progenies consisting of exact genetic replicas of the mother plant. The harnessing of apomixis represents an enormous potential benefit to agriculture, since its introgression into sexual crops would greatly expand the current breeding and seed production capacity. In grass species, this trait is controlled by one or few genetic determinants. Particularly in Paspalum notatum, apomixis segregates a simple dominant locus (i. e. apospory controlling locus or ACL) with a distorted segregation ratio. Comparative mapping analyses revealed that a segment of rice chromosome 12 long arm is consistently syntenic to the Paspalum ACL in at least four species of the genus. This chromosome segment, of about 5.8 cM, likely includes key apomixis governing genes. One of the candidates mapping in this area is LOC_Os12g40770 (OS12G0599900), which encodes an ankyrin (ANK)-containing protein of 423 aa. ANK repeats are present in proteins involved in diverse biological processes such as cell cycle regulation, growth and development and were associated with apomixis in Penissetum ciliare and Poa pratensis. OS12G0599900 encodes a protein with six ANK and two tetratricopeptide (TPR) repeats at the N- and C-terminal regions, respectively. This type of ankyrin (ANK-TPR) is represented by a single member in Arabidopsis (AT3G04710), which encodes a carboxylate clamp-tetratricopeptide repeat (TPR) protein with potential to interact with Hsp90/Hsp70. In rice, the ANK-TPR are represented by 22 members. The objective of this work was to investigate a possible contribution of the Paspalum OS12G0599900 orthologs to the genetic control of apomixis. The OS12G0599900 cDNA sequence was used as query in BLASTx and BLASTp searches onto floral 454/Roche transcriptome databases of apomictic and sexual P. notatum. Ten homologous alleles/splice variants (isotigs), corresponding to six different genes (isogroups), were detected in the apomictic database. The lengths of the transcripts varied from 539 to 1971 bp, while the e-values ranged from e-131 to e-18. In the sexual library eight homologous alleles/splice variants, belonging to seven different genes were detected. The lengths of sequences varied between 733 and 1795 bp. E-values ranged from e^{-130} to $3e^{-38}$. Protein prediction analysis showed that only five out of the 18 isotigs (apoisotigs 11446, 11445 and 20350 and sexisotig 17384, 20570) encoded proteins of the ANK-TPR family (the rest showed homology in the ANK domain only). Four of these sequences (apoisotigs 11446 and 11445 and sexisotig 17384, 20570) mapped in silico within the region of rice chromosome 12 associated with apomixis (by applying the criterion of 65% identity over at least 60% of the length of the sequences and E-values < 0.005). Therefore, they qualified to be Paspalum OS12G0599900 candidate orthologs. We conducted experiments to identify which sequences map onto the ACL by using a segregating family for apomixis of 15 individuals, derived from the sexual tetraploid mother plant (Q4188) and an apomictic pollen donor (Q4117). The reproductive mode of each F_1 plant was determined by cytoembryological observation of cleared ovules at anthesis and by assaying the SCAR marker PNSA2, which generates a band of 180 bp fully linked to the trait. Both parental plants, a sexual bulk (SB) and an apomictic bulk (AB) (5 individuals each) were used for testing the linkage to the ACL. PCR amplification of apoisotig11445 with three allele-specific primer combinations showed polymorphic patterns between the parental plants, but co-segregation with the ACL was not detected. Amplification of apoisotig 11446 showed a monomorphic band, which made segregation analysis impracticable. Amplification of apoisotig 20350 rendered several segregating bands, but none of them mapped at the ACL. The sexisotig 17384 didn´t amplified with the primers designed, and thus new pair of primers should be analyzed. Results obtained so far indicated that at least 6-7 genes (isogroups) encoding for proteins with ANK repeats are being expressed during the P. notatum reproductive development. At least five sequences corresponded to the ANK-TPR family and could represent putative orthologs to the rice gene mapping onto the ACL syntenic region. However, genetic linkage of these sequences with the trait could not be experimentally confirmed yet. Shortly, wide-genome sequencing will be used to investigate the existence of an OS12G0599900-like ankyrin sequence within the Paspalum ACL and its expression in flowers (the latter through comparisons with the 454/Roche transcriptome databases). Moreover, qRT-PCR experiments will be carried out to analyze the ANK-TPR expression pattern during sexual and apomictic reproductive development.

Molecular characterization of a PPIase gene linked to apomixis in *Paspalum notatum*

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Resumen

Paspalum notatum is an apomictic forage grass widely distributed in tropical and subtropical regions of South America. Apomixis is a natural form of asexual reproduction by seeds, which generates progenies genetically identical to the mother plant. This type of reproduction is considered as a deregulation of the sexual developmental pathway, produced by genetic and/or epigenetic factor/s. Apomixis in tetraploid *P. notatum* is controlled by a single locus (ACL) that showed a distorted segregation ratio and restriction in recombination. Molecular analysis have shown that the ACL includes protein coding and non-coding sequences, repetitive elements, and cytosine methylation. Comparative mapping studies showed that markers located at the long arms of chromosome 2 and 12 of rice, mapped completely linked to apomixis in the species. One of these markers (RFLP C932) (LOC_Os02g52290.1 of rice) codifies for a peptidyl-prolyl cis/trans isomerase protein of the FKBP family. This type of proteins are involved in protein folding and has been associated with cell division and cell elongation in *Arabidopsis*. The objective of this work was to characterize the orthologous sequences of locus LOC_Os02g52290.1 in *P. notatum* genome and to determine its relation with the apomictic trait. Tetraploid genotypes Q4188 (sexual), Q4117 (apomictic) and one F_1 family of 21 individuals derived from them were used. The reproduction mode of each F_1 plant was determined by cytoembryological observation of cleared ovules at anthesis, and by assaying the SCAR marker PNSA2, which generates a band of 180 bp completely linked to the trait. Individuals that showed only meiotic embryo sacs in their ovules (*Polygonum* type: egg cell, two polar nuclei and antipodals) were classified as sexual, while individuals showing at least one aposporic embryo sac (egg cell, two polar nuclei and absence of antipodals) were classified as apomicts. For the identification of orthologous PPIase sequence of *P. notatum*, the complete CDS of rice was retrieved from the Gramene web page (www.gramene.org). A BLAST search was carried out against a 454/Roche transcriptome database of the flower development of apomictic and sexual *P. notatum* genotypes in order to identify similar sequences. Afterward, specific PCR primers for each transcript (isotig) were designed for PCR amplification from genomic DNA. Genetic linkage of amplicons with the trait was performed by analyzing both parental genotypes, a bulk of 5 sexual and 5 apomictic F_1 progenies and then all individual samples. The classification of the F_1 individuals by both citoembryological observation and molecular analyses showed 14 sexual and 7 apomictic progenies. BLAST analysis on the 454 database using the rice PPIase as a query detected eight highly similar sequences. In the sexual genotype, five isotigs (alleles/splice variant) of 1,471-3,113 bp corresponding to the same isogroup (gene= isogrup00104) were identified. Among them, the isotig01380 (*S*= 333; *E-value*= $6e^{-91}$) was the most similar to the query in the phylogenetic analysis. The predicted protein (112 aa) was highly similar to the FKBP-type peptidyl-prolyl cis-trans isomerase of *Sorghum bicolor* (XP_002454586.1). In the apomictic genotype, three different sequences (isogroups = genes) were detected. The first one was a short transcript of 631 nt (isotig35750; *S*= 325; *E-value*= $2e^{-88}$) which encoded for a protein similar to the rice gene. The second one (isotig32671; *S*= 82; *E-value*= $4e^{-15}$), seems a truncated form of the PPIase gene, and the third one (isotig33823; *S*= 240; *E-value*= $8e^{-63}$) corresponded to a chimeric transcript containing a FKBP-domain and a DNAJ domain. The specific amplifications of all isotigs determined that only the short version of the rice gene (isotig35750) co-segregates with the ACL. This sequence showed a specific band presents in the apomictic parent and all apomictic progenies that was absent in sexual plants. Moreover, the same band was detected in several other natural apomictic accessions. The band of interest is being cloned and sequenced to confirm its identity. Specific fragments of sexual and apomictic isotigs will be amplified for expression analysis by qRT-PCR during sexual and apomictic reproductive development. Moreover, the complete genomic sequence will be isolated for characterizing the gene structure in both genotypes and analyze the epigenetic landscape associated with both sequences.