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Analysis of mode of reproduction of guayule (*Parthenium argentatum* A. Gray) using flow cytometry and identification of polyhaploids for breeding

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ABSTRACT

Guayule (*Parthenium argentatum* A. Gray) is a promising domestic source of solid rubber for tire manufacturing in North America. Crop improvement and breeding efforts on guayule have enabled improved germplasm to be developed and deployed on commercial fields. One difficulty in guayule breeding is the identification of the mode of reproduction, particularly in polyploids, which exhibit a mixed mode due to their facultative nature. Guayule polyploid plants range from being triploids ($3n = 3x = 54$) to octaploids ($8n = 8x = 144$). Polyploids are known to reproduce by facultative apomixis, while diploids ($2n = 2x = 36$) follow a sexual mode of reproduction.

Flow cytometry is a useful tool for determining ploidy levels of plants. One objective of this study was to estimate the rate of apomixis occurring in different USDA germplasm lines, as well as whether or not there was a meiotic reduction in the megaspore mother cell, and if there was fertilization to form an embryo. We examined the leaf tissue of maternal plants and 100 progeny seeds from these plants, to estimate their rate of apomixis. Combined ploidy information from two different seed tissues (embryo and endosperm) was utilized. Flow cytometric analysis of polyhaploids was also conducted to enable validation of their mode of reproduction, which have not previously been identified in the literature. Results from this study indicated that off-type progeny (seed) would not have been identified if only leaf samples were used, resulting in either, an over-estimation of the rate of apomixis for the accession, or misclassification of the type of reproduction. All identified polyhaploids were analyzed for genetic diversity, and were compared with existing diploid germplasm.

1. Introduction

There are a number of plants that produce rubber. Among these, guayule (*Parthenium argentatum* A. Gray), which was discovered and first described in 1852, has a long history of research and utilization. Guayule is a member of the Asteraceae (Compositae) family, and is related to 16 recognized *Parthenium* species (The Plant List, 2013). Guayule rubber was marketed as early as 1905 from factory scale rubber extractions in Mexico (Lloyd, 1942). Currently, R & D investments on guayule are geared towards it being a viable domestic source of solid rubber for tire production and hypo-allergenic latex. These initiatives on guayule and other alternative crops, such as the Russian dandelion (*Taraxacum kok-saghyz* L.E. Rodin), are to ensure a stable supply or raw material for the industry and diversify commercial sources of natural rubber (Van Beilen and Poirier, 2007).

In support of guayule commercialization and scale up, breeding and agronomic activities seek to increase rubber yields. The available public germplasm for guayule breeding is limited. Currently, there are only two diploids and 40 polyploid accessions that are available (GRIN, 2017; ; Gore et al., 2017). The publically available germplasm materials are fairly narrow in genetic diversity (Ilut et al., 2015; Ray et al., 2005), and breeding activities capitalize on the facultative nature of guayule reproduction which periodically releases new variability in polyploid germplasm (Thompson and Ray, 1989). Progress in the genetic improvement in guayule is evident in the increase in average rubber yield from 220 kg/ha, which was reported during the Emergency Rubber Project in the 1940's, to more than 1000 kg/ha, as seen in the present polyploid germplasm (Hammond and Polhamus, 1965; Ray et al., 1999).

Knowledge on the mode of reproduction and ploidy level of

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germplasm is critical for the continued advancement in guayule breeding. Screening methods to characterize the apomictic potential in guayule have been reported (Keys et al., 2002). Diploids ($2n = 2x = 36$) are known to follow a sexual mode of reproduction, whereas observations on polyploids ($3n \geq 54$) indicate that they are facultative apomicts that show predominantly apomictic reproduction (asexual reproduction by seed) with periodic sexual recombination (Gerstel and Mishanec, 1950), or they can be highly sexual even at high ploidy levels (Gardner, 1946). Tetraploids and triploids are the most common among guayule accessions. Guayule polyploid plants range from triploids ($3n = 3x = 54$) to octaploids ($8n = 8x = 144$) (Thompson and Ray, 1989).

In the past, cytological observations were employed to assess the level of ploidy in guayule (Powers and Gardner, 1945; Jasso de Rodriguez et al., 1993; Herickhoff et al., 1994). The use of flow cytometry for ploidy determination in guayule simplified and greatly shortened the effort of this process. The method has been applied to characterize the ploidy variation of available guayule public germplasm in the US National Plant Germplasm System (NPGS) (Gore et al., 2011). In addition to baseline ploidy determination of nuclear genome size, flow cytometry using seeds have been used in other plant species to determine the mode of reproduction (Matzk, 2007). Flow cytometry was also used to identify desirable cytotypes for breeding (Ochatt, 2008), and also assess seed quality (Sliwinska, 2009). In this study, flow cytometry using guayule seeds was conducted to deduce possible modes of reproduction in guayule to estimate the rate of apomixes and whether or not there was meiotic reduction in the megaspore mother cell and fertilization to form an embryo. The information obtained from flow cytometry seed screening (FCSS) was compared with, or used in conjunction with results from leaf tissues. The study also validated the mode of reproduction in a limited set of polyhaploids, and assessed their utility in guayule breeding. Polyhaploids arise infrequently from reduced and unfertilized tetraploid plants (Rollins and Catcheside, 1951). These appear to have similar ploidy as a diploid plant under flow cytometry, because DNA of somatic cells is used to infer chromosome number and ploidy level. Polyhaploids offer potential for generating new genetic recombinations for plant breeding. Rollins and Cacheside (1951) reported that this type of germplasm resulted in progenies with improved rubber content. Additional information about their mode of reproduction is presented in this study.

2. Materials and methods

2.1. Plant materials

Seeds of guayule and interspecific hybrid accessions R1095 (W6 2247), R1100 (W6 2252), 11604 (PI 478642), CAL 7 (W6 7157), 11635 (PI 478648), and AZ-2 (PI 599675) were obtained from the USDA-ARS National Arid Land Plant Genetics Resources Unit (NALPGRU) in Parlier, CA. Other diploid plants (HS8-1, 13001, 13002, 13007, 13008, and 13013) were from the Bridgestone germplasm collection. The plants were established at the Bridgestone Guayule Research Farm in Eloy, AZ. The guayule seeds were planted in 1020 seedling trays filled with Sunshine mix® (SunGro Horticulture, Agawam, MA) and the seedlings allowed to grow for three months in the greenhouse before transplanting in the field. Different seed maturity levels were obtained using the flower head appearance as an indication of maturity. The determination of the exact number of days required for each stage mentioned in this paper is on-going. Preliminary analyses of seeds from different floral stage were conducted to see if the endosperm ploidy was easier to interpret at a certain development stage. Erickson and Benedict (1947) previously observed that guayule seed endosperm only remained at the micropylar end of the ovule and can be found, at most, as two layers of cells on mature seeds. The majority of the analyses on the seed were done on seeds from late floral stage after preliminary testing. Ripe seeds, based on brown color appearance, were manually collected from a representative plant of each accession when plants were 1-year-old. The seeds were threshed and cleaned prior to them being used for flow cytometry.

2.2. Flow cytometry

2.2.1. Leaves

A nuclei suspension of each accession was made by placing the seeds in a tube with 3.2 mm dia. stainless steel bead and 500 µl extraction buffer (Cystain UV Precise P Extraction Buffer, 2.5 µM polyvinylpyrrolidone (PVP), 10.12 mM DL-Dithiothreitol). A leaf tissue of a diploid guayule accession was incorporated in each sample as an internal standard. Tissue disruption was made using a homogenizer (Tissue Lyser II, Qiagen, CA) at 25 Hz for 19 s, and the sample incubated at room temperature. The suspension was passed through a 30 µm nylon mesh filer (Millipore, Billerica, MA) by centrifugation at 3000 rpm for 10 s, and 400 µl of staining buffer added. The suspension was analyzed by flow cytometry in a CyFlow® Ploidy analyzer (Sysmex, Lincolnshire, IL) equipped with UV LED (365 nm) and green laser (532 nm). Approximately 100 µl of solution was run for each sample.

2.2.2. Seeds

Nuclei suspension of each accession was made by placing the seeds in a Petri plate and 500 µl extraction buffer (Cystain UV Precise P Extraction Buffer, 2.5 µM polyvinylpyrrolidone (PVP), 10.12 mM DL-Dithiothreitol). A leaf tissue of a diploid guayule accession was incorporated in each sample as an internal standard. Tissue disruption was made by manually chopping the samples using a sharp razor blade. An additional 500 µl of extraction buffer was poured to the chopped tissue and the suspension was passed through a 30 µm CellTrics nylon mesh filter (Sysmex, Billerica, MA). The nuclei suspension was stained by adding 400 µl of staining buffer (Cystain UV Precise P Staining Buffer), and by incubating the samples at room temperature. The samples were analyzed by flow cytometry as above.

The flow cytometry software parameters were adjusted using the diploid genotype (2*C*), and the relative fluorescence peak position from this internal standard. The method of reproduction for seed formation was interpreted according to Fig. 1, which were consolidated from Hammond and Polhamus (1965), and Thompson and Ray (1989).

2.3. Pollen fertility

Pollen from anthers of polyhaploid plants was collected at noon and observed using bright field microscopy after staining the pollen with lactophenol cotton blue solution (Sigma-Aldrich, St. Louis, MO). Viable pollen was distinguished by observing for dark blue staining. Non viable pollen stained light blue or remained clear.

2.4. Genotyping

Leaf tissues from polyhaploid plants were obtained along with the samples from representative diploid plants (13013-1, 13001-8, 13002-4, 13008-8, 13007-7) that are part of the Bridgestone germplasm collection. Genotyping was performed using 348 SNP markers previously developed by Bridgestone using the RAD-seq method (Davey and Blaxter, 2010). The allelic data were analyzed by computing a simple dissimilarity coefficient in Darwin v.6 (Perrier et al., 2003) using the formula: $d_{ij} = 1 - \frac{1}{L}\sum_{l=1}^{L}\frac{m_l}{\pi}$, where d_{ij} = dissimilarity between units i and j, L = number of loci, π = ploidy, m_l = number of matching alleles for locus l. The dendrogram was constructed using hierarchical clustering method from the dissimilarity matrix following the UPGMA method.

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