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Research Article

Occurrence and Detection of Chimeral Sectors in Leaf Tissue of Novel *Lolium multiflorum* x *L. arundinaceum* Hybrids

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ABSTRACT

A novel diploid, *Lolium multiflorum* (*Lolium perenne* L. subsp. multiflorum (Lam.) Husnot (syn. *Lolium multiflorum* Lam.) line has been previously observed to induce genome loss and dihaploid generation following hybridization with hexaploid, *L. arundinaceum* (Schreb.) Darbysh.) (Syn.Festuca *arundinacea* Schreb.). Early observation of chimeral sectors in these F1 has suggested a form of mitotic chromosome or genome loss was responsible for the recovery of the *L. multiflorum* or *L. arundinaceum* dihaploids, and that flow cytometry was a methodology that can be utilized to observe such events. Evaluations of leaf tissue nuclei, extracted from 21 F1 hybrids, were submitted to flow cytometry analysis to obtain ploidy estimation within those leaf tissue sections. Results of the analysis suggest a high degree of mitotic genome instability in the leaf tissue sections, providing indicated that mitotic chromosome or genome instability was one cause for dihaploid generation in these materials. A comparison regarding the frequency of sectoring across the various *L. arundinaceum* genotypes, as well as a comparison of greenhouse vs field nursery growing conditions, did not indicate any positive or negative influences.

Key words: Lolium; Hybrids; Flow Cytometry; Chimera; Detection

INTRODUCTION

Flow cytometry is often applied for the estimation of nuclear DNA content or estimate ploidy in plants, especially if the research objective is focused on discerning between 1n, 2n and 4n tissue [1,2]. Chimera sectors in plant tissue occur when an individual's genetic material are separated into distinct zones of separate cell lineages. A number of causes ranging from tissue irradiation to an incompatibility of genomes are typically attributed to such behaviors [3,4]. As is the case with a species such as Hosta sp., chimera tissue can be readily observed by the presence of green and white sectoring in the leaves. In more difficult situations, chimera tissue can be recognized by a variety of methods ranging from biochemical tests, cytogenetics, molecular markers, flow cytometry, etc. [4]. Researchers interested in how the generation of leaf chimera can elucidate apical meristem development and its effects on plant generation have used flow cytometry to identify and verify chimera sectors in leaf tissue of several species, often using embryonic calli [5-8].

A recent approach to induce dihaploids in tall fescue (Lolium arundinaceum) following hybridization with a novel, dihaploid inducing annual ryegrass (*L. multiflorum*) inducer line (IL) has been described [9-11]. This approach provides an alternative to tissue culture methods often utilized to generate dihaploids [12]. One caveat of this alternative tall fescue dihaploid generation approach results in the formation of ryegrass/tall fescue sectors in the original, parental *L. multiflorum* x *L. arundinaceum* F1 hybrids. These sectors often result in the production of multiple leaf sectors that have eliminated either the *L. multiflorum* or the *L. arundinaceum* genome, or perhaps a portion of either genome. This genome loss behavior are suggested to be a result of mitotic loss of the respective genomes, presumably in their entirety, giving rise to pure *L. multiflorum* or *L. arundinaceum* haploid sectors [11]. Following the loss of either the ryegrass or tall fescue genome, the remaining genome within these sectors can spontaneously double, giving rise to recovered diploid (2n=2x=14) *L. multiflorum* or hexaploid (2n=6x=42) *L. arundinaceum* dihaploid recoveries [9-11].

Following the phenotypic observation of chimera sectors in these F1 individuals (Figure 1), the presumption that a previously undefined level of mitotic genome instability among the two genomes was the agent for the chimera sectors. A flow cytometry approach was applied to analyse leaf tissue sections of F1 hybrids to determine if sectoring could be observed and to provide a visualization of the mitotic genome loss process. Attempts to associate the degree of leaf sectoring to dihaploid recovery and phenotypic expression were also investigated.

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Figure 1: An example of chimera sectoring in a *L. multiflorum* x *L. arundinaceum* F1 hybrid. A fertile, 2n=2x=14, *L. multiflorum* sector is on the left; a fertile, 2n=6x=42 *L. arundinaceum* sector is on the right.

MATERIALS AND METHODS

Plant material and preparation

L. multiflorum, inducer lines (IL) were hybridized by pollen from a myriad of tall fescue genotypes [11] (Table 1). Hybrids were sown to pots in the greenhouse and allowed to grow to an intermediate, pre-flowering maturity. In 2012, the leaf samples were removed in a 360° radial pattern around the circumference of each mature F1 hybrid. Earlier experiments and sampling methods on single leaf samples had identified the presence or absence of sectoring leaf tissue; therefore, to obtain a greater understanding of the plants genotype and phenotype with regard to levels of chimera sectoring. It was presumed that this multiple leaf sampling approach would facilitate the understanding of the genome instability behavior and genome loss occurrence in a plant at any given time during sampling. Utilizing this methodology, 21 F1 IL x Lolium arundinaceum plants were reviewed for the detection of their evels of chimeral sectoring (Table 1). The F1 hybrids evaluated utilized six proprietary *L. arundinaceum* germplasm resources and two *L. arundinaceum* cultivars (Barolex and Kora) as the pollen parents. Hybrid seed and *L. arundinaceum* materials were provided by Barenbrug Seeds, USA (Tangent, OR).

Leaf Samples					
Hybrid ID	**TF Genotype**	**Lf1**	**Lf2**	**Lf3**	**Lf4**
228-1*	6FRD	F1/6N	F1/6N	6N	6N
245-2*	Barolex	1N/6N	1N	6N	6N
113-2*	Kora	6N	F1/6N	F1/6N	6N
144-1*	07-Rz80-3	6N	6N	F1	6N
144-2*	07-Rz80-3	6N	6N	1N	F1
144-3*	07-Rz80-3	6N	6N	6N	6N
144-5*	07-Rz80-3	F1/6N	2N/F1/6N	F1/6N	1N/F1
134-1	07-WTD 12-18	F1/6N	F1/6N	6N	1N/6N
135-1	07-WTD 12-18	1N/6N	1N/6N	6N	6N
136-2	07-WTD 12-18	F1/6N	F1/6N	6N	1N/6N
137-2	07-WTD 12-18	1N/6N	6N	F1/6N	6n
138-3	Barolex	1N/6N	F1/6N	F1/6N	6N
139-1	BE5602-39	F1/6N	F1/6N	6N	F1/6N
140-3	BE5602-39	1N/6N	6N	6N	6N
141-2	BE5602-39	6N	6N	F1/6N	F1/6N
143-3	BE5602-39	6N	F1/6N	F1/6N	1N/6N
143-4	BE5602-39	6N	6N	F1/6N	F1
174-2	LT02A	F1	F1	1N/F1	F1
184-1	06-Fa-AmphiBdm-6	F1/6N	F1/6N	F1	F1/6N
185-1	06-Fa-AmphiBdm-6	F1/6N	F1/6N	F1/6N	F1/6N
186-1	06-Fa-AmphiBdm-6	F1/6N	F1/6N	F1/6N	F1/6N
*Greenhouse grown sample					

Table 1: 21 L. multiflorum x *L. arundinaceum* F1 hybrids evaluated by flow cytometry. All the hybrids had either IL1 or IL2 as their L. multiflorum "inducer" parent. Four leaf segments, designated as Lf1, Lf2, Lf3 and Lf4 were sampled. Genome size estimations are indicated in the table as 1x = 7n L. multiflorum, haploid set; F1 = 7n L. multiflorum + 21n L. arundinacum; 6x = hexaploid (2n=6x=42) a *L. arundinaceum* dihaploid genome. Hybrid ID samples having an asterix were greenhouse grown. Hybrid ID samples not having an asterix were field grown individuals.

For the flow cytometric analysis, approximately 0.05 g of fresh cut leaf tissue is placed in 1.5 ml Eppendorf tubes. Approximately 0.05 g of a 0.9-2.0 maceration stainless steel bead product (SS-B14B, Next Advance Inc., Averill Park, NY, USA) and one 3.2 mm stainless steel bead (SSB32, Next Advance Inc., Averill Park, NY, USA) are combined for leaf maceration. 500 ul of Galbraith solution was placed in each tube [1] and each sample was placed in a rotary bullet blender tissue homogenizer (Next Advance Inc., Averill Park, NY, USA) at a setting of 8 and run for 10 mn to macerate the leaf tissue.

Approximately 400 ul of this fluid are transferred from the Eppendorf tubes to 15 ml Corning tubes. Nuclei labelling and detection is achieved by dispensing 1 ml of the FxCycle PI/RNase staining solution (Invitrogen by Thermo Fisher Scientific, 81 Wyman Street, Waltham, MA 02451 USA) into the macerated leaf tissue for one hour. Following the manufactures staining recommendations, samples were retained in darkness during the one hour staining interval. Prior to flow cytometric analysis each sample is filtered through a 50 um CellTrics disposable filter (Sysmex-Partec GmbH, Goerlitz, Germany) before evaluations in a Life Technologies Attune NxT Acoustic Focusing Flow Cytometer (Model AFC2, Thermofisher Scientific, 81 Wyman Street, Waltham, MA 02451 USA).

Flow cytometer settings

Description of FC settings: The flow cytometer was set to deliver a volume of 100 µL from the sample syringe at a flow rate of 25 µL/min. The Attune NxT software was not under control of the user and the software parameters were initiated at the "start" mode for a few seconds, prior to initiating the "record" mode. This step is performed to stabilize the sample rate and equilibrate the concentration of the dye bound to the sample nuclei. The threshold of the forward scattering (FSC) detector was placed in the "or" mode while the side scattering and fluorescence detectors were placed in the "ignore" mode. Default threshold settings were utilized for the logic control box (OR) and the forward scattering channel detector (FSC) was set at a threshold of 25.0 × 1000. The number of recorded events was set to 500 with the particular event peaks being gated and average median fluorescent values are used to estimate the ploidy/genome size of the nuclei being recorded. L arundinaceum cultivars of Nanyro and Drover were used as checks to provide a base line estimator for 2n=6x=42 genome size estimations. The original *L. multiflorum* inducer line IL1, including a series of previously known, well characterized IL × La F1 hybrids were used to establish the various baselines for the *L. multiflorum* and F1 hybrid genome size estimations.

RESULTS AND DISCUSSION

Flow cytometry has been applied to many plant species mostly focused on nuclear DNA content and the estimation of ploidy, often using DNA specific fluorochromes [2]. In this experiment, flow cytometry was performed on leaf tissue to identify the presence of chimeral sectors in a series of novel *L. multiflorum* x *L. arundinaceum* F1 hybrids. Of the 84 leaf samples evaluated in this study, the histogram peaks identifying the DNA content from the flow cytometry analysis were well resolved in 80 of the 84 samples tested (95.2%), with CV values ranging from 2.98% to 6.20% with an average CV of 4.35% (SD=0.68%).

21 Lm × La F1 hybrids were evaluated for this study. Seven were grown in greenhouse conditions and fourteen under field nursery conditions. All 21 hybrids exhibited sectoring through genome loss (Table 1). Flow cytometry genome size estimations were correlated with known standards for genome size and chromosome constitution and were divided into the following genome size groups: 1x=7n, the predicted *L. multiflorum*, haploid genome size; F1=a full 7n *L. multiflorum*+21n L. arundinacum F1 hybrid genome size estimate; 6x=a hexaploid (2n=6x=42), *L. arundinaceum* dihaploid genome size estimate (Table 1).

Flow cytometric results identified seven classes of nuclei within the four leaf samples (Lf1, Lf2, Lf3 and Lf4) across the 21 F1 hybrids (Table 1). The seven classes are identified as F1/6n, 1n/6n, 1n/F1 and 2n/F1/6n sectors and non-sectoring samples exhibiting 1n or 6n nuclei. Of the 84 leaf samples obtained from the 21 F1 plant samples, 43 individual leaf samples exhibited some type of sectoring. Across the 21 F1's sampled, 17 exhibited sectoring in one or more of its leaf samples. Of the three samples that exhibited no sectoring, each clearly reflected a reduction or alteration in their prior F1, 28 chromosome genome constitution, with genome size estimates moving toward an estimated 2n=6x=42 or 1n=1x=7 genomic state. An exceptional example illustrating the ability of flow cytometry to detect genome instability can be seen from data generated for hybrid 144-5 (Table 1) where a leaf sample provided three distinct peaks. One peak being equivalent to a diploid/dihaploid, 2n=2x=14 *L. multiflorum* genome size estimation; a second, central peak having equivalency to a F1 *L. multiflorum* x *L. arundinaceum* hybrid possessing 7 *L. multiflorum* chromosomes and 21 *L. arundinaceum* chromosomes; and a third, smaller peak having a genome size equivalency to a *L. multiflorum* individual with a 2n=6x=42 genome size. It is anticipated if the 2n=2x=14 *L. multiflorum* region and/or the 2n=6x=42 *L. arundinaceum* regions became isolated in a rapidly dividing apical meristem, and spontaneously doubled, a dihaploid *L. multiflorum* or *L. arundinaceum* individual would result.

A comparison regarding the frequency or type of sectoring across the *L. arundinaceum* genotypes or greenhouse vs field nursery conditions, suggested that there were no obvious correlations or difference in an F1 individuals frequency of genome loss or sectoring. The results taken in their entirety indicate a rather robust level of genome instability and genome loss in these novel *L. multiflorum* x *L. arundinaceum* F1 hybrids.

Among the sectoring individuals, peak height varied considerable, suggesting differences in nuclei counts for each particular peak. In some instances, a high number of 1n nuclei was observed as compared to the second peak where a lower peak height indicated fewer nuclei (Figure 2). Utilizing the peak area, one can estimate the particular percentage of the leaf sample that the sectors occupied (Kindiger, unpublished, 2017; data not shown).

Projected haploid *L. multiflorum* individuals (1n=1x=7) exhibited a peak with a fluorescence corresponding to approximately half of that of diploid *L. multiflorum* (2n=2x=14) checks. It is anticipated that following *L. arundinaceum* genome loss, only the *L. multiflorum*, or most of it remains, giving rise to the observed 1n=1x=7 sectors. These sectors can generate *L. multiflorum* dihaploids. Previous identification of *L. multiflorum* dihaploids, strongly support this potential outcome and prior research investigations, indicate dihaploid *L. multiflorum* individuals were generated [13]. Recent molecular studies using SST markers on *L. arundinaceum* recoveries, has homozygous status. Leaf nuclei samples exhibiting a genome size estimation of 2n=6x=42 indicate the potential for

dihaploid generation (Figures 3 and 4). The flow cytometry genome estimations of nuclei from these leaf tissues correspond well with the 2n=6x=42 *L. arundinaceum* checks utilized in the study. Similar market studies in the presumed *L. multiflorum* DH recoveries are anticipated to provide a similar outcome.

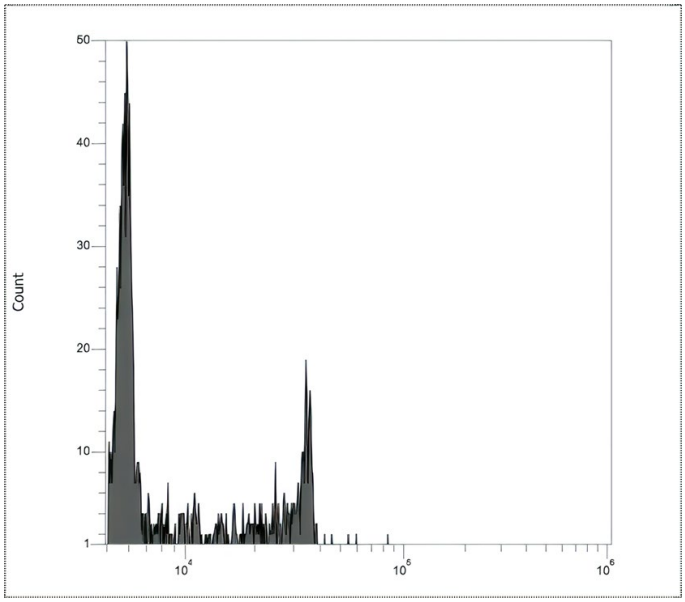

Figure 2: Flow cytometric analysis of leaf tissue nuclei obtained from A *L. multiflorum* (IL) x *L. arundinaceum* F1 hybrid. The larger left peak corresponds to a haploid (n=7) estimated genome size for *L. multiflorum*. The smaller right peak, corresponds to a (2n=6x=42) estimated genome size for *L. arundinaceum*. The relative heights of the peaks provide an estimate regarding the number of nuclei present in the leaf sample and a corresponding indication on the relative size of the leaf sectors. In the above image, the far larger number of 1n=7 nuclei suggest a large haploid sector (left), when compared to the far smaller 2n=6x=42 sector or peak (right).

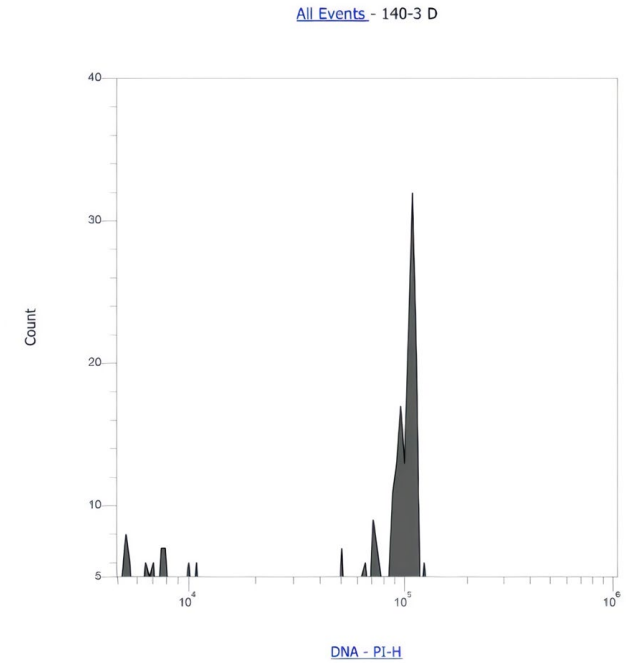

Figure 3: Flow cytometric analysis of leaf tissue that indicates all nuclei in the sample exhibit a full 2n=6x=42 genome constitution. The identification of this type of event suggests loss of the *L. multiflorum* genome, retention of the haploid set of the *L. arundinaceum* genome, followed by spontaneously doubling, providing a leaf segment exhibiting only 2n=6x=42 leaf nuclei. Identification of this dihaploized region strongly suggest the generation of a dihaploid *L. arundinaceum* sector. If this sector can be maintained in the apical meristem region, a *L. arundinaceum* dihaploid vegetative sector may be obtained.

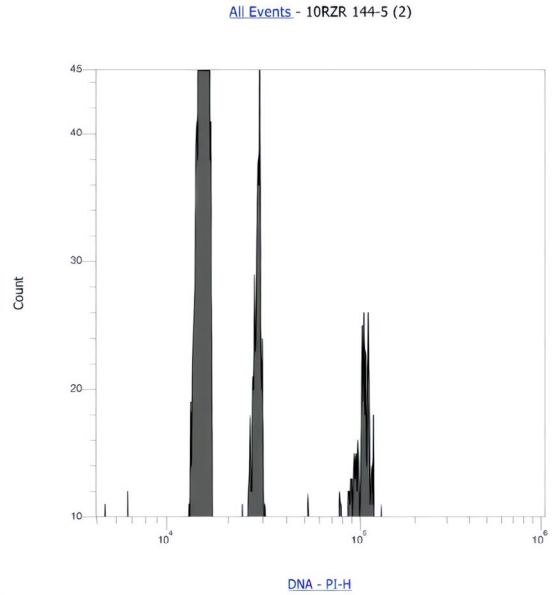

Figure 4: Flow cytometer results of nuclei extracted from a leaf segment of F1 hybrid 144-5 that depicts three distinct peaks, each associated with anticipated genome size estimations. A large peak is observed that is equivalent to a 2n=2x=14 diploid/dihaploid *L. multiflorum* (left); a F1 hybrid peak corresponding to a genome size estimation of nuclei possessing 7 *L. multiflorum* and 21 *L. arundinaceum* chromosomes (center); and a smaller, peak corresponding to a genome size estimation of nuclei equivalent to a 2n=6x=42, dihaploid, *L. arundinaceum* sector.

The flow cytometry evaluations allowed the investigator to visually record and confirm a high level of genome instability in the novel *L. multiflorum* x *L. arundinaceum* hybrids. Flow cytometric analysis was found to be an effective and accurate method for identifying and verifying chimera sectors as induced through genome instability within the leaf samples of these novel F1 *L. multiflorum* x *L. arundinaceum* hybrids. The approach, for the first time, allows in real time, a glimpse of the genome instability behavior in these materials and provides a clearer understanding of the level of mitotic genome instability that occurs in these novel hybrids. The detection of such haploid sectors or dihaploid sectors will effectively improve the selection efficiencies of dihaploid generation [9-11].

CONCLUSION

The successful application of this mitotic genome instability "inducer" hybridization approach provides an attractive alternative to dihaploid generation when compared to traditional microspore culture methods.

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