Analysis of Active Nucleolus Organizing Regions in Polyploid Prairie Cordgrass (\textit{Spartina pectinata} Link) by Silver Staining

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Summary Prairie cordgrass has recently gained attention as an important biotic component of stressed ecosystems. This polyploid species is distributed broadly across the U.S. More cytogenetic data are needed to investigate how increased ploidy levels influence chromosome changes and gene expression in order to understand the formation of stable cytotypes. Silver staining is the cytogenetic method commonly used to study the number and distribution of active nucleolar organizing regions (NORs) on chromosomes at metaphase, and the number and size of nucleoli in nuclei at interphase. Intensity and distribution patterns of silver stained NORs (AgNORs) reveal differential ribosomal gene expressions in plants and animals. The purpose of this study is to estimate the number of AgNORs, their locations, and their activities in metaphase chromosomes and to determine heteromorphic variation in size and number of nucleoli within interphase nuclei for tetra-, hexa-, and octoploid prairie cordgrass populations. Increases in mean numbers of AgNORs in each metaphase and interphase cell were observed as ploidy level increases. Although distribution patterns of silver stained NORs at metaphase cells of tetra- and octoploids reflect changes in ploidy, the neo-hexaploids did not follow the pattern, indicating that active NORs were not stable in early generation of formation of neo-hexaploidy. Collectively, these results suggest that distribution patterns of silver stained NORs in metaphase and number of silver stained nucleoli in interphase cells can be used as markers to detecting chromosome variations within and among ploidy levels and to determine when ploidy levels stabilize in prairie cordgrass.

Key words Prairie cordgrass, Silver staining, Nucleolus organizing region, AgNOR, Polyploidy.

Prairie cordgrass (\textit{Spartina pectinata} Link) is a tall, rhizomatous, perennial, and warm-season grass (C\textsubscript{4}) native to a wide geographic range in North America. This species has recently gained attention as an important biotic component for soil and water conservation practice on agricultural landscapes along with biomass production (Long 1975, Potter \textit{et al}. 1995, Boe and Lee 2007, Montemayor \textit{et al}. 2008, Skinner \textit{et al}. 2009, Boe \textit{et al}. 2009, Kim \textit{et al}. 2012a). Due to its massive rhizome and deep root system (Weaver 1968), prairie cordgrass is able to survive and even thrive under abiotic stress conditions such as waterlogging (Jensen 2006), soil salinity (Montemayor \textit{et al}. 2008, Kim \textit{et al}. 2012a) and drought (Boe \textit{et al}. 2009). In addition, the combination of genomic changes and increased genetic diversity induced by polyploidization (Parisod \textit{et al}. 2010) may be a key factor in growth, performance, adaptability of polyploid prairie cordgrass in such marginal environments.

Prairie cordgrass has been reported as a multi-polyploid species consisting of three ploidy levels: tetraploid (\textit{2n}=40), hexaploid (\textit{2n}=60), and octoploid (\textit{2n}=80) with a basic chromosome number of \textit{x}=10 (Church 1940, Marchant 1968a,b, Kim \textit{et al}. 2010). Kim \textit{et al}. (2012b) studied intraspecific ploidy level variations in 183 prairie cordgrass individuals collected across the U.S. to...
understand the potential relationship between ploidy levels and geographic distribution. According to cytogeographic distribution of prairie cordgrass, the tetraploids extended from the East North Central to the New England regions of the U.S., while the octoploids were mostly distributed in West North Central regions. Hexaploids were only found in one location in which hexaploids were co-occurring with tetraploids in central Illinois (Kim et al. 2012b). Our previous studies indicated strong associations of genetic (Kim et al. 2013) and morphological variation (Guo et al. 2015) with geographic distributions, meaning that genetic diversity found in polyploid prairie cordgrass can be exploited for phenotypic variation and wide geographical adaptation. However, increased genes and genome dosage in polyploids can also cause negative effects such as genome instabilities, chromosome imbalances, regulatory incompatibilities, and reproductive failures (Chen 2007). We need more sufficient cytogenetic and molecular data to investigate how increased ploidy influences the structural changes of chromosome that maintain the stability of cytotypes within their respective distributed area.

Silver staining is the cytogenetic method commonly used to study the number, size, and distribution of silver stained nucleolar organizer regions (AgNORs) at metaphase or nucleoli at interphase. During metaphase, silver atoms deposit onto argyrophilic proteins associated with nucleolar organizer regions (NORs) which are involved in a high rate of production of ribosomal RNA genes and of ribosomes that are necessary for all protein synthesis in the cell (Olert et al. 1979, Buys and Osinga 1980, Jordan 1987, Hubbell 1985). AgNORs are located in secondary constriction of chromosome satellites which is constant in shape and size for each particular chromosome (Mahabal 2010), and the intensity of the silver reaction is reflective of the nucleolar transcriptional activity taking place during the interphase preceding metaphase (Ferraro and Lavia 1983, Schwarzacher et al. 1978). Comparison of the distribution patterns and intensity of AgNORs at metaphase between and within ploidy levels, therefore, can help in evaluating alterations of chromosomal structures and the rate of production of ribosomal materials, which may be involved with growth and performance associated with the stabilization of polyploidy series. In interphase, silver atoms deposit onto the fibrillar centres and the fibrillar components of the nucleolus containing ribosomal DNA (rDNA) and acidic proteins associated with rDNA sites (Howell and Black 1980, Pébusque and Seïte 1981, Paweletz and Risueño 1982, Fernández-Gómez et al. 1982, Sumner 1982). The amount of silver staining in the nucleoli has been shown to correlate with ploidy level and/or proliferative activity of cells (Darvey and Driscoll 1972, Crocker et al. 1998, Sumner 2003).

In this study, six prairie cordgrass (Spartina Pectinata Link) populations were studied: two tetraploids (IL102 and 20-102), two hexaploids (17-116 and 103-6x), and two octoploids (PCG109 and 19-108). Their cytotypes and geographic distribution were already given by Kim et al. (2010, 2012a,b). The objectives of this study were to estimate the number of AgNORs, their locations, and their activities on metaphase chromosomes and to determine the heteromorphic variation in size and number of nucleoli at interphase cells for different ploidy levels in prairie cordgrass populations.

Materials and methods

Plant material

Fresh clones of six populations (2 tetra-, 2 hexa-, and 2 octoploids) of prairie cordgrass were used for this experiment (Table 1). The rhizomes were grown in an aeroponic system and maintained under greenhouse conditions (16-h photoperiod at temperature of 24–26°C).

Silver staining

Fresh young root tips were collected and fixed in ethanol–acetic acid (3:1) after a 2-h
treatment with 0.005% of 8-hydorxyquinolin. The root tips were stored at 4°C for 2 d. The fixed root tips were digested in an enzyme solution (0.2 g Cellulysin and 0.1 g Macerase in 10 mL of 10 mM EDTA, pH 6) for 2.5 h. The root tips were placed on a slide with a drop of 45% acetic acid and were chopped with a thin blade. A cover slip was placed on the tissue and gently tapped with a dissecting needle to disperse all of the tissue. Direct pressure was applied to the slide, and the slide was stored at −80°C until use. The cover slip was removed after freezing and preparation was air-dried for 1.5 h.

Slides were incubated in 2×SSC (300 mM NaCl and 30 mM Na3C6H5O7 in 1 L of ddH2O, pH=7) with coverslips in a humidity chamber at 37°C for 5 min, washed in ddH2O, and air-dried. Slides were covered with 50 μm nylon mesh after adding a few drops of freshly prepared 50% silver nitrate solution in ddH2O. The slide was incubated in a humidity chamber at 60°C for 7 h, washed in ddH2O, and air-dried in the dark for 1 d. The dried slide was soaked in xylene for 5 min. A few drops of permount were added, extra permount was rinsed with xylene, and a coverslip was added. The silver staining was visualized by using an Olympus BX61 microscope. Photographs were taken using an Olympus U-CMAD3 camera. Image analysis software (Image J) was used to estimate the number and size of Ag-positive dots and size of nucleus from 100 randomly selected nuclei per each population.

**Feulgen staining**

Fresh young root tips of "IL102" were collected and stored in 3:1 ethanol/acetic acid for 24 h at 4°C. The fixed root tips were rinsed in ddH2O, hydrolyzed in 5 M HCl for 45 min, and placed in Feugen’s stain for 2 h. Root tips were rinsed in ddH2O and incubated in enzyme solution (0.2 g Celluysin and 0.1 g Macerase in 10 mL of 10 mM EDTA) for 1 h at 37°C. The enzyme solution was washed with 45% acetic acid. Root tips were squashed onto slides in a drop of 45% acetic acid. The slides were viewed using an Olympus BX61 microscope. Photographs of nuclei were taken using an Olympus U-CMAD3 camera.

**Results**

The six populations of prairie cordgrass analyzed in the present work showed the following chromosome numbers: 2n=40 ("IL102" and "20-102"), 2n=60 ("17-116" and "103-6x"), and 2n=80 ("PCG109" and "19-108") (Table 1).

<table>
<thead>
<tr>
<th>Line</th>
<th>Ploidy level (x=10)</th>
<th>Origin</th>
<th>References</th>
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<tbody>
<tr>
<td>IL102</td>
<td>4x</td>
<td>IL</td>
<td>Kim <em>et al.</em> 2010</td>
</tr>
<tr>
<td>20-102</td>
<td>4x</td>
<td>KS</td>
<td>Kim <em>et al.</em> 2012b</td>
</tr>
<tr>
<td>103-6x</td>
<td>6x</td>
<td>IL</td>
<td>Kim <em>et al.</em> 2012a</td>
</tr>
<tr>
<td>17-116</td>
<td>6x</td>
<td>IL</td>
<td>Kim <em>et al.</em> 2012b</td>
</tr>
<tr>
<td>PCG109</td>
<td>8x</td>
<td>SD</td>
<td>Kim <em>et al.</em> 2010</td>
</tr>
<tr>
<td>19-108</td>
<td>8x</td>
<td>IW</td>
<td>Kim <em>et al.</em> 2012b</td>
</tr>
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The silver stained active nucleolar organizer regions (AgNORs) observed at metaphase are visualized as black spherical bodies on yellow-brown chromosome arms (Fig. 1). The number and position of AgNORs varies among six prairie cordgrass populations. In "IL102" three black silver precipitates were found near one side of telomeric reigions: two AgNORs located on both chromosome arms and the other located on only one chromosome arm (Fig. 1A). In "20-102" three chromosomes showed AgNORs located in telomeric regions on both their arms, and one chromosome has an AgNOR located on only one chromosome arm (Fig. 1B). "17-116" has six
AgNORs bearing chromosomes which possess four AgNORs located in telomeric regions on both chromosome arms and two AgNORs located in the pericentromeric region on only one side of the chromosome arms (Fig. 1C). "103-6x" possesses two AgNORs located in telomeric regions on both chromosomes arms and one AgNOR located in the pericentromeric region on one side of chromosome arm (Fig. 1D). Eight AgNORs were found in "PCG109", consisting of two AgNORs located in telomeric regions on both chromosome arms, one located in the pericentromeric region on both chromosome arms, one located in the pericentromeric region on one chromosome arm, and four located in telomeric regions on one chromosome arm (Fig. 1E). "19-108" shows seven AgNOR bearing chromosomes showing four AgNORs located in telomeric regions on both chromosome arms, one located in the pericentromeric region on one chromosome arm, and two located in telomeric regions on one chromosome arm (Fig. 1F). Unlike tetra- and hexaploids, several small AgNORs were observed on either the end or pericentrometric region of a single chromosome arm for octoploid populations.

Two types of interphase cells were observed in root tips: one located in the meristematic region and the other located in the root cap region (Fig. 2). The nucleolus of the cap cell is much smaller than the nucleolus of the meristem cell, but a higher number of nucleoli per cell were

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**Fig. 1.** The number of active NORs observed in mitotic metaphase chromosomes of prairie cordgrass: "IL102" (2n=40) with three active NOR sites (A); "20-102" (2n=40) with four active NOR sites (B); "17-116" (2n=60) with six active NOR sites (C); "103-6x" (2n=60) with three active NOR sites (D); "PCG109" (2n=80) with eight NOR sites (E); "19-108" (2n=80) with seven active NOR sites (F). Arrow indicates the AgNORs. The AgNOR bearing chromosome pairs are shown in the box.
observed in the cap cell (Fig. 2). In order to confirm the presence of nucleolus in interphase cell, a few squash preparations of "IL102" were stained according to the Feulgen technique (Fig. 3). The Feulgen staining is highly specific for DNA, so the results show that the regions in the nucleolus mostly containing rRNA and proteins turn a pale pinkish color, reflecting a lack of DNA (Fig. 3). The distribution of cells with different numbers of nucleoli per interphase nucleus located in both meristem and cap cells and the percentage of interphase cells with heteromorphic nucleoli are shown in Table 2. The mean number of nucleoli varies among ploidy levels. In meristematic interphase cells, the tetraploid population mostly has one large nucleolus per cell, but higher ploidy populations have two to four nucleoli found at low frequency (0.33–9.83%) (Table 2 and Fig. 2A, 2D, and 2G). In tetraploid cap cells, the mean of number of nucleoli per interphase cells of "IL102" and "20-102" are 2.26 and 2.04, respectively, and both populations have mostly two nucleoli per cell (Fig. 2B and 2C). The mean number of nucleoli per interphase cap cells of "17-116" and "103-6x" are 2.66 and 2.76, respectively. In most cases two and three nucleoli per nucleus in cap cells

Fig. 2. The number of silver stained nucleoli of interphase nucleus located in meristem (A, D, and G) and root cap cells (B, C, E, F, H, and I) in prairie cordgrass: "IL102" (2n=40) with one nucleoli in meristem cell (A) and with two nucleoli in root cap cell (B); "20-102" (2n=40) with two nucleoli in root cap cell (C); "17-116" (2n=60) with two nucleoli in meristem cells (D) and with three and four nucleoli in root cap cells (E); "103-6x" (2n=60) with three nucleoli in meristem cell (F); "PCG109" (2n=80) with three nucleoli in root cap cells (H). "19-108" (2n=80) with three and four nucleoli in meristem cells (G) and with six nucleoli in root cap cell (I). The arrow indicates nuclear envelop (N) and nucleolus (NC).
were present (Fig. 2E and 2F), although occasionally more than three nucleoli were also observed. A different distribution of nucleoli per interphase cap cells was observed within octoploids. The “19-108” population has the highest mean number of nucleoli per cap cell, 3.95, and in most cases between 2 and 4 nucleoli per cap cell were present (Fig. 2I), with a maximum of seven nucleoli per cap interphase cell, while the “PCG109” population (Fig. 2H) has a similar mean number of nucleoli per cap cell as hexaploids because this population has mostly two nucleoli per cap interphase cell. The higher mean number of nucleoli has resulted in the higher frequency of cells with silver stained nucleoli heteromorphism. For example, “19-108” has the highest mean number of nucleoli observed at a higher frequency in meristem and cap cell as well was the highest percentage of heteromorphic silver stained nucleoli (8.19% and 70.41%).

Discussion

In the present study, we have shown the successful use of silver staining to visualize active nucleolar organizer regions (NORs) on mitotic chromosomes and evaluate the number, size, and distribution pattern in six prairie cordgrass population with different ploidy levels. Moreover, the
number of silver stained nucleoli were counted per interphase nucleus in both meristem and cap cells, and we have found that interphase nucleoli count correlates with ploidy.

According to our observation, variability of the metaphase AgNOR pattern and the number, locations (Fig. 1), and size of silver-stained nucleoli during interphase (Fig. 2), were observed among and within ploidy levels. The size and intensity of AgNORs localized in secondary constrictions have been correlated with the activity of rRNA genes which are transcribed during the previous interphase (Hubbell 1985, Guerra 2000). The size of silver granule dots at metaphase is much smaller than the silver granule dots at interphase because only 10% of the silver stained nucleolar protein during interphase are retained on metaphase chromosomes (Sumner 2003). The AgNOR pattern in the metaphase cell is specific and constant (Sozansky et al. 1985, Isakova 1994), and thus can be used as the chromosomal marker to detect polymorphisms of Ag-stained NORs on prairie cordgrass chromosomes.

Although two populations within each tetra- and octoploidy have similar numbers of active NOR sites on metaphase chromosomes, two hexaploid populations have different numbers of AgNORs. Unlike the “17-116” population, only three out of six NOR sites were normally stained in the “103-6x” population. A plausible explanation for this result is that three AgNORs localized on chromosome arms that were normally active and visible while the other three additional sets of NORs which may also exist, were functionally inactive during the preceding interphase (Sumner 1990, Wachtler and Stahl 1993). Moscone et al. (1995) and Bustamante et al. (2014) reported that the AgNOR site is correlated with the 45S rDNA site where breaks and gaps caused chromosome fragmentation, resulting in new chromosomal rearrangements. Thus, the loss of nucleolar activity of the NOR observed in the “103-6x” population may be a result of rapid changes in chromosomal structures and gene expressions which may be due to the formation and establishment of neo-polyploidy in mixed ploidy populations co-occurring with tetraploids. This similar result has been observed in allotetraploid Arabidopsis suecica, reporting that some loci in newly formed ploidy are unstable to cause loss, gain, and rearrangement of NOR sites (Pontes et al. 2004). Silver-stained NORs on mitotic chromosomes are therefore useful in determining the stabilization of newly formed polyploidy of prairie cordgrass.

The application of staining technique to interphase nuclei results in deposition of silver atoms in nucleoli, corresponding to the fibrillar centres and fibrillar component which contains ribosomal DNA and acidic proteins associated at the rDNA sties (Hernandez et al. 1980, Fakan and Hernandez-Verdun 1986, Hubbell 1985). In the present study, two types of interphase cells were observed: one located in the meristem region and the other located in the cap cell. Various numbers of nucleoli in both cell types were detected within a population, which can be explained by the fact that the quantity of interphase nucleoli progressively increases from early G1 phases and reaches the maximum value at S and G2 phases (Mourad et al. 1997). In addition, differences in the size and the maximum number of nucleoli were observed between meristem and cap cell. Due to the process of nucleolar fusion, most meristematic interphase cells were found to contain one large nucleolus, but some contained two, three, or four nucleoli at low frequencies. After late telophase the number of small silver stained granules decreases whereas the size of larger granules increases, so interphase nucleoli recover their elliptical shape (Anastassova-Kristeva 1977, Ploton et al. 1987). In contrast, the size of the nucleolus in cap cells was much smaller than in the meristem cells, and higher maximum number of nucleoli involving with high heteromorphic variations of size of nucleoli was observed in cap interphase cells. The maximum number of nucleoli in the cap cells was equal to active AgNORs at metaphase in the samples analyzed, with some exceptions. This observation can be explained by Jordan et al. (1982) who reported that smaller nucleoli have low possibility of fusing, which may increase the number of nucleoli per cell showing several patterns of nucleolar fusion associated with the various combinations of fused and unfused nucleoli in interphase cell.
In general, the mean number of nucleoli increased with increase in ploidy level. The mean number of nucleoli in cap cell of tetra-, hexa-, and octoploids were approximately 2.15, 2.72, and 3.36, respectively. According to previous studies (Wilkinson 1944, Sen and Bhadrui 1968), a multiplication of chromosome sets is invariably associated with a proportionate increase in the number of nucleoli in interphase cell. Thus, the number of nucleoli in cap cell can be used as a reliable marker for ploidy level particularly where mitosis chromosome size is too small to obtain preparations in which most of the cells are in a dividing stage to enable count chromosomes of each cell. Moreover, the high heteromorphic variations in the size of nucleoli were observed in higher ploidy levels. This feature is common in polyploid plants such as wheat (*Aegilops speltoides*, Darvey and Driscoll 1972) and ryegrass (*Lolium multiflorum*, Bustamante et al. 2014). Variation in number of nucleoli is also observed within ploidy level, but not in hexaploids. Unlike other ploidy populations, two hexaploids were found in mixed ploidy populations located in a single location in IL (Kim et al. 2012b), so they showed similar nucleolar fusion patterns. Jordan et al. (1982) reported that the fusion depends on the length of cell cycle, so longer cell cycle increases the possibility of fusion resulting in lower mean number of nucleoli per cell. Based on our results, “19–108” has a higher mean number of nucleoli per cell, reflecting more frequent cell division, and therefore a lower possibility of nucleolar fusion than the “PCG109” population.

**Conclusion**

Using a modified, highly reproducible silver staining procedure, we have studied the variation in the maximum number of AgNOR at metaphase and of nucleoli at interphase cells of tetra-, hexa-, and octoploid prairie cordgrass populations. All prairie cordgrass had from three to eight AgNORs per metaphase cell, and a variation of AgNOR patterns was found among ploidy levels. All populations within each ploidy level, except hexaploidy, have similar numbers of active NOR sites on metaphase chromosomes. Variation in number of active NORs was observed among two hexaploids, suggesting that the active NORs are unstable due to rapid changes in chromosome structure and gene expression caused by formation of neo-polyploidy in mixed ploidy populations. Since the AgNORs detected on mitotic chromosomes are constant and species-specific, they can be used as markers to detect chromosome variations within and among ploidy levels and may play a critical role in determining their chromosomal changes which may affect the gene expressions involving both formation and establishment of polyploidy. The mean number of silver stained nucleoli increases from tetra-to octoploids; therefore the quantitative analysis of silver stained nucleoli per interphase cell should be sufficient to determine the ploidy levels in prairie cordgrass. In addition, silver staining active NORs helps to determine the stabilization of neo-polyploidy in prairie cordgrass. However, further cytogenetic analysis such as fluorescence *in situ* hybridization (FISH) is needed to provide markers that allow individual chromosome pairs to be distinguished, and then to trace the structural changes in chromosomes of newly formed polyploids of prairie cordgrass.

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