Variation in Genome Size, Ploidy, Stomata, and rDNA Signals in Althea

Jason D. Lattier[1]
USDA-ARS U.S. National Arboretum, Floral and Nursery Plants Research Unit, 10300 Baltimore Avenue, Building 010A, Beltsville, MD 20705; Oak Ridge Institute for Science and Education, 100 ORAU Way, Oak Ridge, TN 37830; and Department of Horticulture, Oregon State University, 4017 Agriculture and Life Sciences Building, Corvallis, OR 97331

Hsuan Chen and Ryan N. Contreras
Department of Horticulture, Oregon State University, 4017 Agriculture and Life Sciences Building, Corvallis, OR 97331

ABSTRACT. Althea (***Hibiscus syriacus***) is a shrub prized for its winterhardiness and colorful summer flowers. Altheas are tetraploids ($2n = 4x = 80$); however, breeders have developed hexaploids and octoploids. Previous studies report anatomical variation among polyploids, including stomata size. The purpose of this study was 4-fold. First, identify genome size and ploidy variation in cultivars via flow cytometry and chromosome counts. Second, create a ploidy series consisting of $4x$, $5x$, $6x$, and $8x$ cytotypes. Third, investigate the ploidy series for variation in stomatal guard cell lengths, stomatal density, and copy number of fluorescent ribosomal DNA (rDNA) signals. Fourth, investigate segregation patterns of rDNA signals in a subset of pentaploid seedlings. Flow cytometry revealed most cultivars to be tetraploid with holoploid 2C genome sizes from 4.55 ± 0.02 to 4.78 ± 0.06 pg. Five taxa ('Aphrodite', 'Pink Giant', 'Minerva', Azurri Satin®, and Raspberry Smoothie™) were hexaploids (6.68 ± 0.13 to 7.05 ± 0.18 pg). Peppermint Smoothie™ was a cytochimera with tetraploid cells (4.61 ± 0.06 pg) and octoploid cells (8.98 ± 0.13 pg). To create pentaploids, reciprocal combinations were made between hexaploid 'Pink Giant' and tetraploid cultivars. To create octoploids, seedlings were treated with agar solutions containing 0.2% colchicine or 125 μM oryzalin. Guard cell lengths were significantly different among the four cytotypes: $4x$ (27.36 ± 0.04 μm), $5x$ (30.35 ± 1.28 μm), $6x$ (35.59 ± 0.63 μm), and $8x$ (40.48 ± 1.05 μm). Measurements of stomatal density revealed a precipitous decline in average density from the $4x$ cytotype (398.22 ± 15.43 stomata/mm²) to $5x$ cytotype (194.06 ± 38.69 stomata/mm²) but no significant difference among $5x$, $6x$, and $8x$ cytotypes. Fluorescent in situ hybridization (FISH) revealed an increase in 5S and 45S rDNA signals that scaled with ploidy: $4x$ (two 5S + four 45S), $6x$ (three 5S + six 45S), and $8x$ (four 5S + eight 45S). However, pentaploid ($5x$) seedlings exhibited random segregation of rDNA signals between the $4x$ and $6x$ cytotypes, including all six possible combinations (two 5S, three 5S) × (four 45S, five 45S, six 45S).

Hibiscus is a genus in the Malvaceae comprising ≈250 species of mostly tropical and subtropical trees, shrubs, and herbs divided into 10 sections (Fryxell, 1988; Van Laere et al., 2007). However, few species extend their natural range into temperate climates. Rose of sharon or althea has been a staple ornamental shrub in American gardens and other temperate regions, prized for its winterhardiness, range of flower colors, and unique flower traits, including single-flowered, double-flowered, and semidouble types (Contreras and Lattier, 2014).

The basic chromosome number of *Hibiscus syriacus* has been reported as $x = 20$, with most being tetraploid, $2n = 4x = 80$ (Skovsted, 1941). However, confusion persists as some reports claim *H. syriacus* to be a natural diploid. Van Laere et al. (2007) addressed this discrepancy confirming that "diploid" often is used instead of the correct term "tetraploid." This has led to confusion in classifying hexaploid cultivars (so-called triploids) and octoploid cultivars (so-called tetraploids). A recent draft genome revealed high copy numbers in numerous genes compared with closely related diploid species, confirming *H.*

syriacus as a tetraploid (Kim et al., 2017). Although the majority of taxa investigated have been tetraploid, higher ploidy levels have been reported including hexaploids ('Aphrodite', 'Diana', 'Helene', 'Minerva', 'Melrose', 'Pink Giant', and 'Shimsan') and octoploids ('Purple CV', 'Purple CV_2', 'Red Heart CV', 'Sp$_1$', Sp$_2$, and 'Sd$_1$') (Eeckhaut et al., 2004; Egolf, 1970, 1981, 1986, 1988; Lee and Kim, 1976; Shim et al., 1993; Van Huylenbroeck et al., 2000; Van Laere et al., 2006, 2007, 2009).

Spindle-fiber inhibitors (SFIs) have been used to double chromosomes in *Hibiscus*. Contreras et al. (2009) treated meristems of seedlings with an agar solution containing oryzalin to induce autoallooctoploids of *Hibiscus acetosella*. Li and Ruter (2017) soaked seedlings in colchicine and oryzalin solutions to induce autopolyploids in *Hibiscus moscheutos*. Hexaploids 'Aphrodite', 'Diana', 'Minerva', and 'Helene' of *H. syriacus* were created at the U.S. National Arboretum by crossing improved selections with colchicine-treated seedlings of 'William R. Smith'. Ploidy of the hexaploids 'Melrose' and 'Pink Giant' remained unreported before a flow cytometry survey by Van Huylenbroeck et al. (2000). Researchers in Korea used SFIs and cross-pollination to create hexaploid 'Shimsan' and octoploids 'Purple CV', 'Purple CV_2', 'Red Heart CV', 'Sp$_1$', 'Sp$_2$', and 'Sd$_1$' (Lee and Kim, 1976; Shim et al., 1993). Van

Laere et al. (2006) created hexaploid lines of blue-flowered *H. syriacus* derived from colchicine-treated seedlings of 'Blue Bird' and 'Woodbridge'. Although colchicine has proven effective, no study exists demonstrating the effectiveness of oryzalin in chromosome doubling of *H. syriacus*.

Ploidy levels and chromosome numbers in *Hibiscus* have been confirmed in previous studies using combinations of flow cytometry and root squashes (Contreras et al., 2009; Shim et al., 1993; Van Huylenbroeck et al., 2000; Van Laere et al., 2006, 2009). In addition, anatomical differences among polyploids have been investigated. Observations of morphological characters have consistently shown "gigas" effects among polyploids, including larger flowers, increased flowering duration, and reduced seed production (Egolf, 1970, 1981; Van Huylenbroeck et al., 2000). In *H. acetocella*, Contreras et al. (2009) recorded differences in plant height, leaf size, internode length, canopy volume, pollen diameter, and guard cell lengths among polyploids. In *H. syriacus*, Lee and Kim (1976) recorded differences in leaf thickness, length of guard cells, diameter of pollen grains, and wood fiber anatomy. Shim et al. (1993) reported differences in leaf width, leaf length, flower size, eyespot size, pollen spine number, and pollen exine apertures across ploidy in *H. syriacus*. Van Laere et al. (2009) investigated pollen diameter differences and triad formation among polyploids of *H. syriacus*. Polyploidy also has been hypothesized to effect abiotic stress responses in woody plants, with few studies on drought stress (Li et al., 2009; Pustovoitova et al., 1996) and no current studies on cold-tolerance.

Not all morphological characters are effective at determining genome size and ploidy changes in plants. One reliable character is stomatal guard cell size and density. Genome size has been shown to have a positive correlation with stomata size and a negative correlation with stomata density across a wide range of angiosperms (Beaulieu et al., 2008). Stomata have proven useful anatomical characters to differentiate ploidy levels in woody plant species (Joly and Bruneau, 2007; McGoey et al., 2014; Padoan et al., 2013). Stomatal guard cells have proven a robust enough character that they have been useful for predicting ploidy in dried herbarium specimens (Buechler, 2000; Chen et al., 2009; Saltonstall et al., 2007), and even fossilized leaves (Buechler, 2000). Stomata measurements and anatomical descriptions have been reported for many species of *Hibiscus* (Contreras et al., 2009; Essiett and Iwok, 2014; Li and Ruter, 2017; Zhuang and Song, 2005), yet only one study has reported an association between ploidy and stomata variation in *H. syriacus* (Lee and Kim, 1976). Guard cell lengths of tetraploid controls ranged from 28 to 29 μm, whereas those of colchicine-induced octoploids ('Sp$_1$', 'Sp$_2$', and 'Sd$_1$') ranged from 32 to 42 μm. However, there are no confirming studies and no data on interploid cytotypes, such as pentaploids or hexaploids.

Confirming ploidy level in woody plants can be achieved with flow cytometry and traditional cytology, such as root squashes. Root squashes can be difficult and tedious as many woody plants possess small, friable roots with numerous, small chromosomes (Lattier et al., 2013; Ochatt, 2008). Fluorescent labeling of rDNA could be used to confirm ploidy levels in a ploidy series with numerous, small chromosomes, as well as provide a tool for investigating chromosome segregation through copy number variation in rDNA signals of interploid hybrids.

rDNA has been used to study the origin and evolution of plant genomes in ancient allopolyploids (Volkov et al., 2017) as well as artificial autopolyploids (Gomes et al., 2014) and interploid hybrids (Wang et al., 2015a). Tandem repeated rDNA units are highly conserved throughout all plants and often are combined with more variable, rapidly evolving intergeneric spacer regions (Volkov et al., 2017). Frequently used loci include the 5S and 45S (Ribeiro et al., 2008; Volkov et al., 2017). The 45S and 5S rDNA loci are usually located at different sites on different chromosomes, and their transcription is carried out by different RNA polymerases (Srivastava and Schlessinger, 1991). These markers have become widely used in FISH, a technique that fluorescently labels rDNA and allows for comparison of copy number and location. FISH has proven an efficient technique for cytological studies in woody angiosperms where karyotyping is limited due to the small size of chromosomes (Anamthawat-Jónsson, 2003; Prado et al., 1996). No data exist on rDNA signals in *Hibiscus*.

The objectives of this study were 1) to identify genome size and ploidy variation in cultivars of *H. syriacus*; 2) to create a ploidy series consisting of 4*x*, 5*x*, 6*x*, and 8*x* cytotypes using a combination of interploid hybridization and autopolyploid induction via colchicine and oryzalin; 3) to investigate the ploidy series for variation in stomatal guard cell length, stomatal density, and copy number of fluorescent rDNA signals; and 4) to investigate segregation patterns in rDNA signals in a subset of pentaploid seedlings.

Methods and Materials

Plant material. Plants of *H. syriacus* were collected from nurseries, gardens, and arboreta to represent a cross section of available cultivars in the nursery trade (Table 1). Plants were maintained in containers at the Lewis Brown Farm at Oregon State University (Corvallis). Original cultivar and trademark names were maintained from each source (Table 1); however, usually one name becomes common in the nursery trade as the market name. For simplicity, only market names (cultivar or trademark) will be used hereafter.

Flow cytometry. Holoploid (2C) relative genome sizes were recorded for each accession. For each plant, three expanded leaves were collected randomly to represent three samples of nuclei for each accession. A single leaf was sampled from additional clones of each taxon. If genome size variability was found among additional "clones," then three leaves were screened and additional genome sizes were reported for those accessions, as in *H. syriacus* 'Aphrodite' (Table 1). For each leaf sample, an internal standard of known genome size was included [*Solanum lycopersicum* 'Stupicke' (2C = 1.96 pg)]. Combined leaf tissues (1–2 cm^2) representing a sample plus internal standard were co-chopped in 400 μL of a buffer solution (Cystain Ultraviolet Precise P Nuclei Extraction Buffer; Sysmex, Görlitz, Germany). The resulting solution was poured through a 30-μm gauze filter (Celltrics; Partec, Münster, Germany) into a 3.5-mL plastic tube (Sarstedt Ag & Co., Nümbrecht, Germany) followed by 1.6 mL of fluorochrome stain [4′,6-diamidino-2-phenylindole (DAPI); Cystain Ultraviolet Precise P Staining Buffer; Partec]. The nuclei suspension was analyzed using a flow cytometer (CyFlow Ploidy Analyzer; Partec) with a minimum of 3000 nuclei analyzed per sample at a cv for each histogram less than 10. References to genome size and ploidy follow the terminology proposed by Greilhuber et al. (2005). Holoploid (2C) genome size was calculated as:

J. Amer. Soc. Hort. Sci. 144(2):130–140. 2019.

131

Table 1. Ploidy and relative holoploid 2C genome size in cultivars of *Hibiscus syriacus*.

Cultivar	Trade name[z]	Accession no.[y]	Ploidy[x]	Holoploid 2C genome size [mean ± SE (pg)]	Source[w]
Aphrodite	'Aphrodite'	11-0215	6x	7.05 ± 0.18 a[v]	Bailey Nurseries
Pink Giant	'Pink Giant'	11-0217	6x	6.97 ± 0.07 ab	Bailey Nurseries
Minerva	'Minerva'	13-0051	6x	6.86 ± 0.03 ab	Blue Heron
DVPazurri	Azurri Satin®	14-0188	6x	6.81 ± 0.04 ab	Spring Meadow
DS03RS	Raspberry Smoothie™	14-0094	6x	6.68 ± 0.13 b	Greenleaf Nursery
Antong Two	Lil' Kim™	12-0021	4x	4.78 ± 0.06 c	Bailey Nurseries
DS02SS	Strawberry Smoothie™	14-0091	4x	4.73 ± 0.07 c	Greenleaf Nursery
Woodbridge	'Woodbridge'	11-0214	4x	4.68 ± 0.04 c	Bailey Nurseries
Blushing Bride	'Blushing Bride'	13-0059	4x	4.68 ± 0.04 c	Monrovia
Ardens	'Ardens'	13-0050	4x	4.67 ± 0.05 c	Blue Heron
Notwoodtwo	White Chiffon®	13-0044	4x	4.66 ± 0.04 c	Blue Heron
Aphrodite	'Aphrodite'	11-0215[u]	4x	4.66 ± 0.03 c	Bailey Nurseries
Mineru	Tahiti™	12-0024	4x	4.66 ± 0.06 c	Bailey Nurseries
Mathilde	Blush Satin®	13-0058	4x	4.66 ± 0.03 c	Monrovia
Buddha Belly	'Buddha Belly'	14-0128	4x	4.66 ± 0.05 c	Yamaguchi Nursery
Notwoodone	Lavender Chiffon™	13-0046	4x	4.64 ± 0.03 c	Blue Heron
Notwoodthree	Blue Chiffon™	13-0056	4x	4.63 ± 0.02 c	Monrovia
Collie Mullins	'Collie Mullins'	13-0061	4x	4.63 ± 0.03 c	Monrovia
Bluebird	'Bluebird'	11-0219	4x	4.63 ± 0.04 c	Monrovia
Minspot	Fiji™	12-0022	4x	4.63 ± 0.03 c	Bailey Nurseries
Marina	Blue Satin®	11-0210	4x	4.62 ± 0.03 c	Bailey Nurseries
Lucy	'Lucy'	11-0216	4x	4.62 ± 0.04 c	Bailey Nurseries
Minsygrbl1	Hawaii™	12-0020	4x	4.62 ± 0.04 c	Bailey Nurseries
Minfren	Bali™	12-0023	4x	4.61 ± 0.00 c	Bailey Nurseries
Minerva	'Minerva'	11-0213	4x	4.61 ± 0.03 c	Bailey Nurseries
DS04PS	Peppermint Smoothie™	14-0093	4x + 8x	4.61 ± 0.06 c | 8.98 ± 0.13	Greenleaf Nursery
DS01BS	Blueberry Smoothie™	14-0092	4x	4.60 ± 0.04 c	Greenleaf Nursery
Red Heart	'Red Heart'	11-0212	4x	4.60 ± 0.03 c	Bailey Nurseries
JWNfour	Pink Chiffon®	13-0067	4x	4.60 ± 0.04 c	Monrovia
Bricutts	China Chiffon™	13-0060	4x	4.59 ± 0.04 c	Monrovia
Diana	'Diana'	11v0211	4x[u]	4.58 ± 0.02 c	Bailey Nurseries
American Irene Scott	Sugar Tip®	12-0019	4x	4.55 ± 0.02 c	Bailey Nurseries

[z]Most common name in nursery trade.

[y]Accession number in the Ornamental Plant Breeding Laboratory at Oregon State University (Corvallis). Multiple replicates from the same source under the same accession number.

[x]Ploidy level inferred from genome size estimate.

[w]Container plant collected from the following sources: Bailey Nurseries (Yamhill, OR), Blue Heron Farm (Corvallis, OR), Greenleaf Nursery (Park Hill, OK), Monrovia (Dayton, OR), Spring Meadow Nursery (Grand Haven, MI), Yamaguchi Plantsman Nursery (Gifu, Japan).

[v]Minimum significant difference of 0.31 based on Tukey's honestly significant difference test.

[u]Chromosomes counted using root tip cytology.

$$2C = DNA\ content\ of\ standard \times \frac{mean\ fluorescence\ value\ of\ sample}{mean\ fluorescence\ value\ of\ standard}.$$

CYTOLOGY. To calibrate genome sizes from flow cytometry with ploidy levels, a root squash was performed on a tetraploid cytotype following the protocol of Lattier et al. (2017). Metaphase chromosomes were screened at magnification ×200 using a compound light microscope (Axio Imager A1; Zeiss, Oberkochen, Germany). Fifteen highly resolved cells were imaged under oil immersion at a magnification of ×1000. Chromosomes were imaged at different focal distances and focus-stacked using the Auto Blend utility in Photoshop CC 2015.5.1 (Adobe Systems, San Jose, CA). Greater ploidy levels (5x, 6x, and 8x) were confirmed using root squashes combined with rDNA signal variation from FISH analysis (details to follow).

PLOIDY SERIES. From 2013 to 2015, interploid hybridization and induced autopolyploidy were used to create a ploidy series (4x, 5x, 6x, and 8x). Interploid hybridizations were performed between a hexaploid taxon, 'Pink Giant', and a suite of tetraploid cultivars. A total of 935 crosses were performed with 'Pink Giant'. When using 'Pink Giant' as a seed parent, 379 crosses were attempted representing 19 different combinations. When using 'Pink Giant' as a pollen parent, 556 crosses were attempted representing 24 different combinations. Controlled crosses were performed during summer in a glasshouse kept free of pollinators with day/night set temperatures of 25/20 °C and a 16-h photoperiod. Dried capsules were collected before dehiscence in fall. Seed from each cross were directly sown into 1.3-L containers filled with a peat-based medium (Metro-Mix; Sun Gro Horticulture, Agawam, MA) and seeds were evenly spaced at 30 or fewer seeds per pot. Seedlings from each successful cross were screened using flow cytometry. A vigorous selection with an

intermediate, pentaploid genome was selected for the ploidy series, H2013-078-01 ('Pink Giant' × Bali™).

To create octoploid cytotypes, germinating seedling meristems were treated with SFIs at different concentrations and durations. Open-pollinated (OP) seeds were collected from 'Aphrodite' and Bali™ and sown into 1.3-L containers filled with peat-based medium (Metro-Mix). Replicates of five pots with subsamples of 15 seeds per pot were used for each treatment. Pots were grown under cool-white fluorescent lights at 90 mmol·m⁻²·s⁻¹ at 22 to 25 °C with a 16-h photoperiod.

As seedlings germinated, they were treated with two SFIs [oryzalin (Surflan AS; United Phosphorous, King of Prussia, PA) and colchicine (Sigma-Aldrich, St. Louis, MO)] in four treatment combinations: 125 µM oryzalin for 5 d, 125 µM oryzalin for 10 d, 125 µM oryzalin for 20 d, and 0.2% colchicine for 10 d. Colchicine at 0.2% was previously reported effective (Van Laere et al., 2006), yet efficacy of oryzalin has not been reported for *H. syriacus*. In addition, a negative control was included (SFI-free agar droplet for 10 d). Droplets were applied daily after cotyledons expanded to provide access to the apical meristem. Containers were covered with clear humidity domes to maintain treatment droplets. Containers were held in non-draining plastic trays and seedlings were subirrigated as needed. To improve cellular penetration, 1% (v/v) dimethyl sulfoxide (Sigma-Aldrich) was added to the colchicine treatment. To congeal all treatments and maintain droplets on the meristems over the course of each day, 0.55% agar (w/v) (Sigma-Aldrich) was added to each treatment.

After treatments, 13 germinated seedlings from each container were transplanted into individual 0.95-L containers and randomized on a glasshouse bench. As plants grew, necrosis at the treatment sites necessitated immediate cutting propagation. Single and two-node cuttings were taken from many seedlings. Due to the slow recovery post rooting, leaf samples could not be collected for flow cytometry. Therefore, several developing roots were collected from a subset of treatment plants and a single sample was analyzed for each via flow cytometry to identify putative octoploids. A vigorous selection (OP2014-19) with an octoploid genome size resulting from the 125-µM oryzalin 5-d treatment was selected for the ploidy series. The following year, young expanded leaves were analyzed via flow cytometry to confirm the ploidy level of this selection. The final ploidy series comprised four accessions: Bali™ (4*x*), H2013-078-01 'Pink Giant' × Bali™ (5*x*), 'Pink Giant' (6*x*), and OP2014-19 oryzalin-treated OP Bali™ seedling (8*x*).

Stomata measurements. In Summer 2016, four clonal plants representing the ploidy series were growing in the same environment at the Lewis Brown Farm. Three mature leaves for each plant were selected from random branches. Similar-sized leaves were selected about three to four nodes basipetal to the developing meristem. For each leaf, an area between the midrib and first primary vein on the abaxial leaf surface was treated with a thin coat of clear, nitro-cellulose fingernail polish. Strips of clear packing tape were applied to the nail polish. After nail polish dried, the tape strips were removed carefully with forceps. The resulting cuticle peels containing relief impressions of stomata were mounted to microscope slides. Each slide represented a random leaf sample and was treated as a replicate for further analysis. Each slide was viewed with a compound light microscope (Axio Imager A1) at a magnification of ×200.

Images were captured randomly across the microscope slides (AxioCam 105 Color; Zeiss) and processed using image analysis software (AxioVision; Zeiss). Five to 10 images totaling 97 to 289 stomata per slide were used to measure stomatal guard cell length for each cytotype. Total stomata measured (2281) for each cytotype were 799 (tetraploid), 433 (pentaploid), 524 (hexaploid), and 525 (octoploid). All stomata were measured using the line measurement tool (AxioVision). Stomata per slide were treated as subsamples and stomatal guard cell lengths were averaged for each slide. Average guard cell lengths per slide were averaged for a total of three reps per cytotype. For stomatal density, 50 randomly captured images per slide were analyzed for a total of 150 images per cytotype. Total stomata counted (21,015) for each cytotype were 9029 (tetraploid), 4400 (pentaploid), 4095 (hexaploid), and 3491 (octoploid). The number of stomata was counted in each image and an average stomata number per slide was calculated. Average stomata counts for each slide were averaged for a total of three reps per cytotype. To report stomatal density (stomata per square millimeter), each stomata count was multiplied by 6.62 to scale up from the frame of view at a magnification of ×200 (449.2 µm × 336.5 µm) per the following formula:

$$Stomatal\ density\ (stomata\ per\ square\ millimeter)$$
$$= stomata\ count \times \frac{1}{(449.2\ \mu m \times 336.5\ \mu m) \times 1e^{-6}}.$$

After guard cell lengths and stomatal densities were calculated, stomatal index was calculated according to Li et al. (1996):

$$Stomatal\ index = stomata\ length\ (micrometers)$$
$$\times stomata\ density\ (stomata\ per\ square\ millimeter)$$

Photomicrographs of cuticle peels from each cytotype were produced from layered images composed of multiple focal distances viewed at a magnification of ×630. Images were layered using the Auto Blend feature in Photoshop. Average stomata estimates (as well as ploidy levels) were subjected to analysis of variance and means were separated using Fisher's least significant difference [α < 0.05 (SAS Studio; SAS Institute, Cary, NC)].

FISH. Synthesis of probes for FISH was carried out per Chang et al. (2009). A plasmid DNA construct from *Triticum aestivum*, p*TA794* (Gerlach and Dyer, 1980), containing the 5S rDNA repeat (410 bp) was labeled with digoxigenin (DIG-11-dUTP) by nick translation (Roche Diagnostics, Mannheim, Germany). The digoxigenated probe was detected using Texas red (antidigoxigenin-rhodamine Fab fragments) (14877500; Roche Diagnostics). Another plasmid DNA from *T. aestivum*, p*TA71* (Gerlach and Bedbrook, 1979), containing ≈9 kb of coding sequences from the 45S rRNA gene was labeled with biotin (Biotin-16-dUTP) by nick translation (Roche Diagnostics). The biotinylated probe was detected using fluorescein antibiotin (SP-3040; Vector Laboratories, Burlingame, CA). Counterstaining was performed with DAPI suspended in a mounting medium at 1.5 µg·mL⁻¹ (Vectashield; Vector Laboratories).

Roots tips for FISH were collected from softwood cuttings of tetraploid Bali™, hexaploid 'Pink Giant', octoploid OP2014-19, and a random sample of putative pentaploids from interploid crosses. Root tip pre-fixative, fixative, and enzyme digestion steps follow the protocol of Lattier et al. (2017). After digestion, the enzyme solution was wicked away using

low-lint tissue (VWR International, Radnor, PA) and roots were squashed. Dried slides were treated for 15 min in diluted Giemsa stain (Sigma-Aldrich) and were screened for condensed chromosomes at a magnification of ×200 on a light microscope (Axio Imager A1). Slides with condensed chromosomes were selected for FISH. Preparation for FISH analysis was carried out per previous methods (Chen et al., 2015; Chung et al., 2008). Prepared slides were viewed in the dark on a compound microscope (Axio Imager A1) with a fluorescent light source (X-Cite 120Q; Excelitas Technologies, Waltham, MA). At least five metaphase cells with clear rDNA signals were observed for each taxon. Images of fluorescent DAPI, 5S, and 45S signals were captured separately (AxioCam MRm; Zeiss) and images were combined using image analysis software (AxioVision).

Results and Discussion

FLOW CYTOMETRY. Significant differences were found among the taxa investigated for holoploid 2C genome size ($P < 0.01$). Most taxa investigated were tetraploid with genome sizes ranging between 4.55 ± 0.02 pg in Sugar Tip® to 4.78 ± 0.06 pg in Lil' Kim™ (Table 1). No statistical difference in genome size was found among the tetraploid cultivars (Table 1). Ploidy and chromosome numbers were confirmed for the tetraploid group by root tip counts of 'Diana' (4.58 ± 0.02 pg) at $2n = 4x = 80$ (Fig. 1), in contrast to previous reports of 'Diana' being a hexaploid (Egolf, 1970; Shim et al., 1993; Van Huylenbroeck et al., 2000). Other putative hexaploids from the U.S. National Arboretum were found to be tetraploid in contrast to former reports of hexaploidy (Egolf, 1986, 1988), including a single accession of 'Minerva' (4.61 ± 0.03 pg) and a single accession of 'Aphrodite' (4.66 ± 0.03 pg) (Table 1).

Five accessions were found to have genome sizes significantly larger than the tetraploid taxa (Table 1). 'Aphrodite', 'Minerva', and all accessions of 'Pink Giant', Azurri Satin®, and Raspberry Smoothie™ proved to be hexaploids (Table 1). Holoploid genome sizes ranged from 6.68 ± 0.13 pg in Raspberry Smoothie™ to 7.05 ± 0.18 pg in 'Aphrodite'. Having two cytotypes of 'Aphrodite' and 'Minerva' in the cultivar collection could represent a reversion to the tetraploid state over many years of propagation. However, nursery practices, including sexual propagation of cultivars, cultivar substitution, mislabeling, and seedling invasion of stock plants, have been shown to degrade cultivar collections in previous studies (Fantz, 1994). Confirming a previous report by Van Huylenbroeck et al. (2000), 'Pink Giant' was found to be a hexaploid at 6.97 ± 0.07 pg (Table 1). Flower color and form were similar for hexaploids 'Aphrodite', 'Pink Giant', and 'Minerva' (single, pink flowers). However, Azurri Satin® represents the first single, blue-flowered hexaploid *H. syriacus*, produced by Van Laere et al. (2006). Notably, with the inclusion of the pink, double-flowered Raspberry Smoothie™, we report for the first time a hexaploid, double-flowered *H. syriacus*. Depending on their fertility, Azurri Satin® and Raspberry Smoothie™ may offer new opportunities to breeders seeking sterile hybrids with novel floral phenotypes via interploid hybridization.

In addition to tetraploid and hexaploid cytotypes, one accession, Peppermint Smoothie™, was found to be a cytochimera, with both tetraploid cells (4.61 ± 0.06 pg) and octoploid cells (8.98 ± 0.13 pg) and represents the only mixoploid currently reported (Table 1). If the LII histogenic layer is
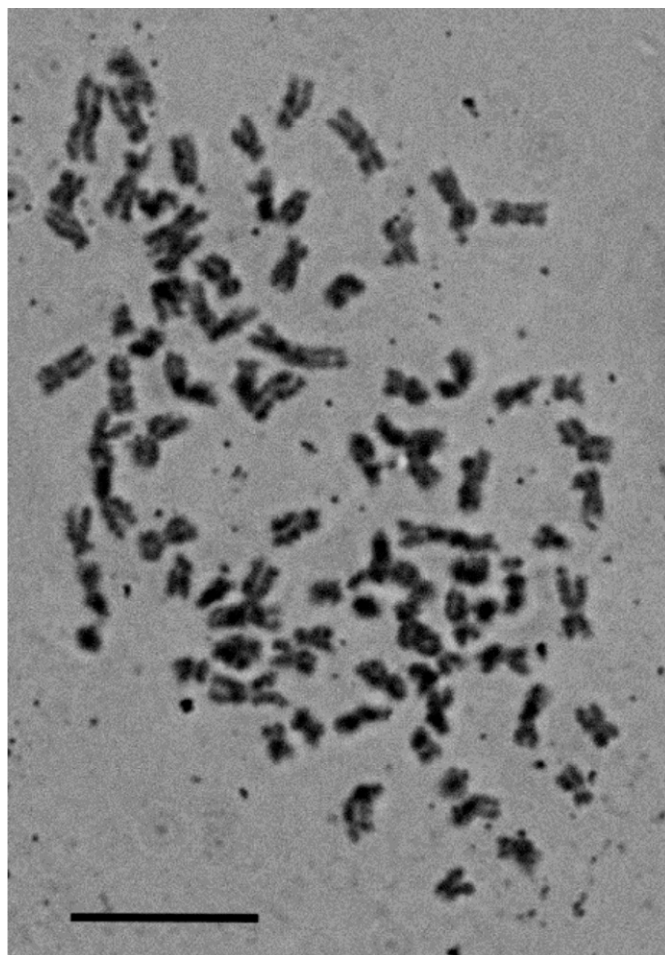

Fig. 1. Photomicrograph of metaphase chromosomes from root tip cells of *Hibiscus syriacus* 'Diana' ($2n = 4x = 80$) viewed at ×1000; scale bar = 10 μm.

octoploid, this cultivar may prove useful for creating novel hexaploids for further breeding.

PLOIDY SERIES. After 935 reciprocal crosses between tetraploid cultivars and 'Pink Giant', 112 capsules were recovered containing a total of 564 seeds. Viable seedlings were recovered from 16 combinations and 'Pink Giant' proved successful as both a seed parent and pollen parent. A subset of these combinations was evaluated with flow cytometry, which revealed numerous seedlings with intermediate (putative pentaploid) genome sizes (Fig. 2). Only one cross, 'Woodbridge' × 'Pink Giant', yielded a near-tetraploid average genome size (4.94 ± 0.19 pg) (Fig. 2) likely due self-pollination. However, one seedling (H2012-041-01), resulting from this cross had a hexaploid genome size of 6.78 pg in a single estimate from flow cytometry. Another cross, *H. syriacus* Bali™ × 'Pink Giant' consistently gave pentaploid genome sizes. Four full-sib seedlings were tested using flow cytometry, yielding an average genome size of 5.70 ± 0.04 (Fig. 2). One vigorous seedling (H2013-078-01), representing this cross was selected for the ploidy series. This seedling had an intermediate genome size (5.55 pg) between its two parents and was included as a representative pentaploid in further analysis of stomata anatomy and copy number variation of rDNA signals.

During the octoploid induction experiment, treatments with SFIs appeared to leave the meristem undamaged and intact
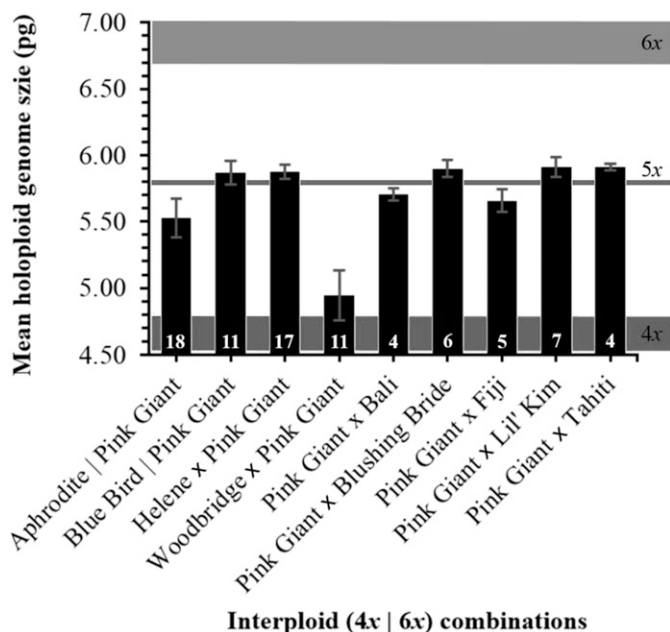
Fig. 2. Flow cytometry estimates of average holoploid 2C genome sizes in a subset interploid crosses between tetraploid *Hibiscus syriacus* cultivars and hexaploid *H. syriacus* 'Pink Giant'. Reciprocal combinations indicated by "|" symbol, whereas "x" represents a unidirectional cross. Vertical bars represent mean ± SE for each cross. White letters within vertical bars represent the number of seedlings measured for each cross. Horizontal $4x$ bar represents the range of tetraploid genome sizes found among cultivars of *H. syriacus*. Horizontal $6x$ bar represents the range of hexaploid genome sizes found among cultivars of *H. syriacus*. Horizontal $5x$ line represents the theoretical pentaploid genome size based on an average of $4x$ and $6x$ genome sizes.

treatment with SFIs to young seedlings caused the tissue death near the treatment site. Subsequent cuttings were taken acropetal to the treatment site and rooted under mist. Thirty-four plants were recovered from the autopolyploid induction experiment, representing a fraction of the original seedling population. Before potting the cuttings, adventitious roots were collected for analysis via flow cytometry. Adventitious roots provided only enough material for a single run on the flow cytometer per sample without proving too damaging to the young cuttings. Samples represented the L-III histogenic layer from which adventitious roots are derived.

Of the remaining plants, only two 'Aphrodite' OP seedlings treated with colchicine produced shoots that rooted. Flow cytometry of roots revealed seedlings OP2014-27 and OP2014-35 had genome sizes of 8.37 pg ($8x$) and 12.22 pg ($\approx 10x$), respectively. No colchicine-treated seedlings of Bali™ remained for genome size analysis. Two 10-d and 20-d oryzalin treatments of 'Aphrodite' OP seedlings were recovered for flow cytometry analysis of roots, which had genome sizes consistent with tetraploids. The remaining 30 accessions were all recovered from 5-d oryzalin treatments, with 18 representing 'Aphrodite' OP seedlings and 12 representing Bali™ OP seedlings. At least one representative from each of the original five replicates was recovered for flow cytometry analysis of roots. For the 'Aphrodite' OP seedlings, 13 seedlings (72%) were found to be octoploid with an average genome size of 8.36 ± 0.11 pg. For the Bali™ OP seedlings, 11 seedlings (92%) were found to be octoploids with an average genome size of 8.36 ± 0.22 pg. Although few seedlings were recovered from the original experiment and only one sample per seedling was analyzed using flow cytometry, our results indicate that colchicine and oryzalin provide an effective way to produce octoploids.

From this autopolyploid population, one Bali™ OP seedling (OP2014-19) from the 5-d oryzalin treatment was selected for further flow cytometry analysis on leaf tissue, yielding an average genome size of 8.88 ± 0.01 pg. This seedling was selected as a representative ocotoploid for the ploidy series and for further analysis of stomata anatomy and variation in rDNA signals.

As expected, holoploid genome sizes from leaf nuclei were different among the four taxa [$4x$, $5x$, $6x$, $8x$ ($P < 0.01$)]. Cell size, including stomata, increased with increasing ploidy (Fig. 3). Guard cell diameter was significantly different among the four taxa in the ploidy series ($P < 0.01$) and ranged from 27.36 ± 0.04 μm in the tetraploid to 40.48 ± 1.05 μm in the octoploid. These results confirm previous reports that stomata size could be useful in distinguishing ploidy in different species of *Hibiscus* (Contreras et al., 2009; Zhuang and Song, 2005). In contrast to stomata in *H. syriacus*, stomata guard cell lengths reported in *Hibiscus schizopetalus*, *Hibiscus mutabilis*, and *Hibiscus rosa-sinensis* were much smaller, ranging from 14.26 ± 0.39 μm to

during germination and subsequent flushes of new growth. However, several weeks after potting, necrosis began to appear on nearly all seedlings. Plants produced healthy shoots post-treatment only to later spontaneously abscise at the treatment site. Control plants appeared unaffected, indicating that
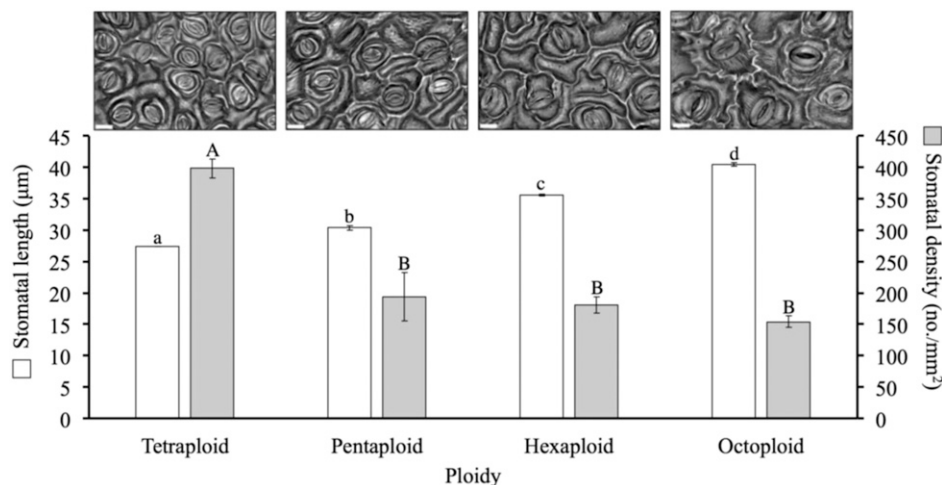

Fig. 3. Stomata length and density in a ploidy series in *Hibiscus syriacus*. Photomicrographs above bar graph are representative cuticle peels for each ploidy level viewed at ×630 magnification (scale bar = 10 μm). Ploidy series included tetraploid *H. syriacus* Bali™ (12-0023), pentaploid hybrid *H. syriacus* 'Pink Giant' × Bali™ (H2013-078-01), hexaploid *H. syriacus* 'Pink Giant' (11-0217), and an octoploid, oryzalin-treated, open-pollinated seedling from *H. syriacus* Bali™ (OP2014-19).

21.10 ± 0.65 µm, respectively (Zhuang and Song, 2005). Essiett and Iwok (2014) reported lengths for *H. acetocella*, *H. rosa-sinensis*, *Hibiscus surattensis*, and *Hibiscus arnottianus* similar to our measurements in *H. syriacus*. In induced autotpolyploids of *H. acetocella* 'Panama Red', guard cell size increased from 25 µm in the tetraploid to 36 µm in the octoploid. Our results are also comparable with a previous report on tetraploid and colchicine-induced octoploid *H. syriacus*. Lee and Kim (1976) found that average guard cell lengths of control tetraploid *H. syriacus* ranged from 28 to 29 µm whereas octoploid guard cell lengths ranged from 32 to 42 µm. Li and Ruter (2017) observed average guard cell lengths in *H. moscheutos* ranged from 24 µm for diploids to 32 µm for tetraploids (J. Ruter, personal communication). These results combined with the current study indicate that guard cell length may be useful for evaluating both interploid and interspecific hybrids in *Hibiscus*.

Significant differences were found in stomatal density among the four taxa in the ploidy series ($P < 0.01$) and ranged from 398.22 ± 15.43 stomata/mm² in the tetraploid to 154.01 ± 8.90 stomata/mm² in the octoploid. There was a significant, precipitous decline in stomatal density from the tetraploid to pentaploid cytotype, representing a 2-fold difference in stomata density. However, no significant differences were detected among the pentaploid, hexaploid, and octoploid cytotypes. A plot of stomatal guard cells sizes and stomatal densities illustrates the negative relationship between guard cell length and density (Fig. 3). The positive relationship between genome size and stomata size, as well as the negative relationship between genome size and stomatal density, proposed by Beaulieu et al. (2008), was observed in the *H. syriacus* ploidy series.

Stomatal index has proven a useful anatomical character for comparing taxa because it is independent of the environment, size, or portion of the leaf surface (Essiett and Iwok, 2014). However, measures differ depending on if total epidermal cell counts are performed, as in Essiett and Iwok (2014), vs. the method used in the current study (Li et al., 1996) that solely relies on the product of the stomata length by the stomata density. For the ploidy series in *H. syriacus*, significant differences were found in stomatal indices among the four taxa investigated ($P < 0.01$). Average stomatal index was $10,894.45 \pm 411.95$ for the octoploid, 5810.05 ± 995.15 for the hexaploid, 6411.81 ± 355.68 for the pentaploid, and 6215.92 ± 196.89 for the tetraploid taxa. The strong effect from the change in stomatal density from tetraploid to pentaploid cytotype resulted in the same trend in the pairwise comparisons among ploidy levels. Polyploids in *Betula papyrifera* also were found to have smaller stomatal indices compared with the diploid controls (Li et al., 1996). Therefore, the reduction in stomatal index in *H. syriacus* at greater ploidy levels may lead to an increase in stress tolerance by reduced transpiration. This effect has been claimed in woody plants but has been illustrated only in herbaceous taxa such as the drought-tolerant autopolyploids in *Arabidopsis thaliana* (Del Pozo and Ramirez-Parra, 2014) and drought-tolerant allopolyploids in *Triticum* (Xiong et al., 2006). The ploidy series of *Hibiscus syriacus* may prove useful in future research on physiological differences across ploidy in woody plants.

FISH. The number of signals from the 5S rDNA locus varied from two (in the tetraploid) to four (in the octoploid), whereas signals from the 45S rDNA locus varied from four (in
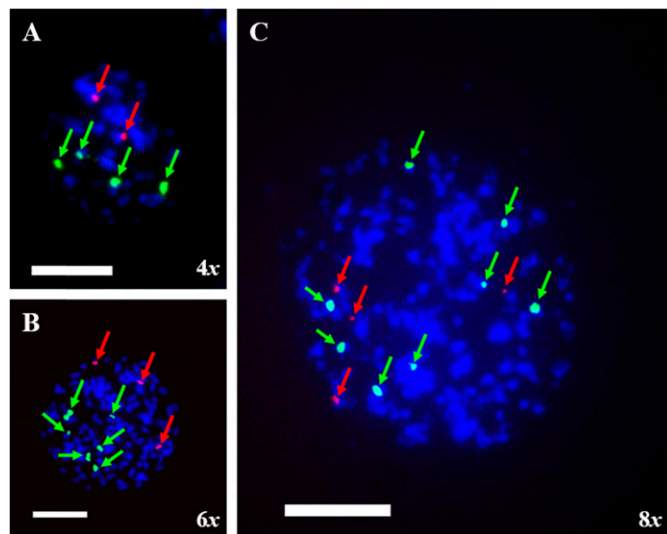

Fig. 4. Fluorescent in situ hybridization (FISH) analysis of metaphase, root tip chromosomes in a ploidy series of *Hibiscus syriacus*. Chromosomes displaying variation in 5S (red) and 45S (green) rDNA loci (scale bar = 10 µm). (**A**) tetraploid *H. syriacus* Bali™ (two 5S signals + four 45 signals). (**B**) hexaploid *H. syriacus* 'Pink Giant' (three 5S signals + six 45S signals). (**C**) oryzalin-treated (125 µM for 5 d), octoploid seedling from *H. syriacus* Bali™ (four 5S signals + eight 45S signals).
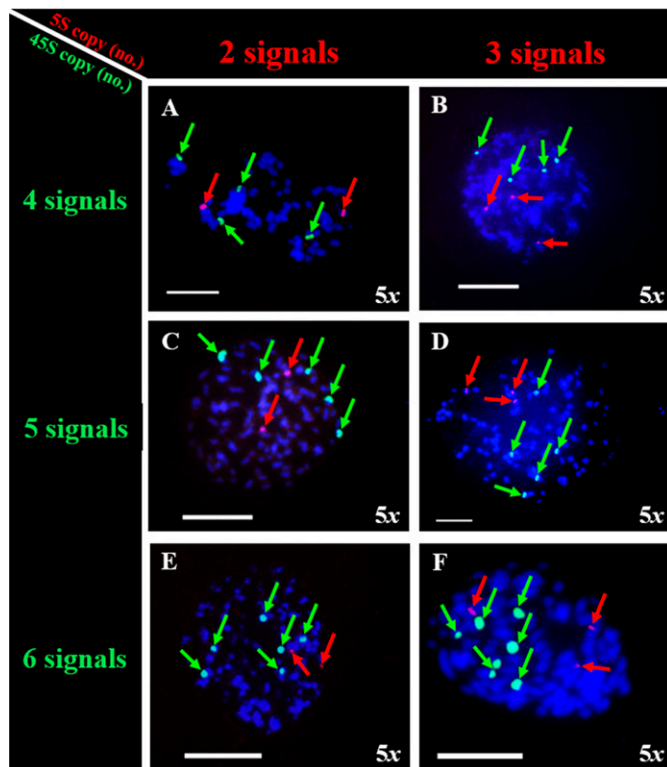

Fig. 5. Fluorescent in situ hybridization (FISH) analysis of metaphase, root tip chromosomes in pentaploid seedlings of *Hibiscus syriacus*. Chromosomes displaying variation in 5S (red) and 45S (green) rDNA loci (scale bar = 10 µm). (**A**) H2013-078-01 'Pink Giant' × Bali™. (**B**) H2013-131-06 'Pink Giant' × 'Blushing Bride' (**C**) H2013-124-19 'Helene' × 'Pink Giant'. (**D**) H2013-017-21 'Aphrodite' × 'Pink Giant'. (**E**) H2013-085-02 'Pink Giant' × 'Red Heart'. (**F**) H2013-049-01 'Diana' × 'Pink Giant'.

136

J. Amer. Soc. Hort. Sci. 144(2):130–140. 2019.

Table 2. Summary of fluorescent in situ hybridization (FISH) analysis in a ploidy series of *Hibiscus syriacus*.

Taxa[z]	Accession no.[y]	Holoploid 2C genome size [mean ± SE (pg)][x]	Ploidy[w]	5S rDNA signals (no.)[v]	45S rDNA signals (no.)[v]
Bali™	12-0023	4.61 ± 0.00	$4x$	2	4
'Pink Giant' × Bali™	H2013-078-01	5.65[u]	$5x$	2	4
'Pink Giant' × Lil' Kim™	H2013-084-16	6.03	$5x$	2	4
'Aphrodite' × 'Pink Giant'	H2013-017-11	5.81	$5x$	2	4
'Helene' × 'Pink Giant'	H2013-124-19	5.89	$5x$	2	5
'Pink Giant' × 'Red Heart'	H2013-085-02	6.07	$5x$	2	6
'Pink Giant' × Fiji™	H2013-129-08	5.64	$5x$	3	4
'Pink Giant' × 'Blushing Bride'	H2013-131-06	5.97	$5x$	3	4
'Pink Giant × 'Aphrodite'	H2013-077-05	5.64	$5x$	3	5
'Blue Bird' × 'Pink Giant'	H2013-044-03	5.60	$5x$	3	5
'Aphrodite' × 'Pink Giant'	H2013-017-21	5.70	$5x$	3	5
'Helene' × 'Pink Giant'	H2013-124-03	6.31	$5x$	3	6
'Diana' × 'Pink Giant'	H2013-049-01	5.71	$5x$	3	6
'Pink Giant'	11-0217	6.97 ± 0.07	$6x$	3	6
Open-pollinated Bali™ (oryzalin-treated seedling)	OP2014-19	8.88 ± 0.01	$8x$	4	8

[z]Taxa represent tetraploid Bali™, hexaploid 'Pink Giant', an octoploid oryzalin-treated, open-pollinated seedling of Bali™, and a range of pentaploid progeny recovered from reciprocal crosses with 'Pink Giant'.
[y]Accession number of parent taxa, hybrids, and experimental open-pollinated seedlings.
[x]Flow cytometry estimates based on leaf samples. Three leaf samples were evaluated for the $4x$, $6x$, and $8x$ cytotypes. A single leaf sample was evaluated for the $5x$ cytotypes.
[w]Ploidy level inferred from genome size estimate.
[v]Number rDNA signals determined using FISH.
[u]Single leaf samples analyzed by flow cytometry among a large population of pentaploid seedlings to determine putative pentaploids for FISH.
rDNA = ribosomal DNA.

the tetraploid) to eight (in the octoploid) (Fig. 4). The use of rDNA signals, combined with flow cytometry, proved useful for confirming ploidy levels in *H. syriacus*, a species with numerous small chromosomes. The discovery of only two 5S rDNA signals in tetraploids (Fig. 4A) could provide evidence for *H. syriacus* being a diploid, an allotetraploid with disomic segregation (functional diploid), or an ancestral autopolyploid with subsequent elimination of rDNA sites. However, it is unlikely that *H. syriacus* originated from a recent autopolyploid event, as four 5S rDNA signals would have been expected.

A recent genome analysis of *H. syriacus* confirms its polyploid status ($2n = 4x = 80$) after multiple occurrences of whole-genome duplication followed by diploidization after speciation (Kim et al., 2017). In addition, allopolyploidy is common in other species of *Hibiscus*. Polyploidy has been investigated in *Hibiscus* section *Furcaria* with tetraploids, hexaploids, octoploids, and decaploids all exhibiting allopolyploidy (Menzel and Wilson, 1969; Wilson, 1994, 1999). Tetraploids of section *Furcaria* have been discovered to be allopolyploids including *H. acetosella* (AABB, $2n = 4x = 72$) and *Hibiscus radiatus* (AABB, $2n = 4x = 72$) (Satya et al., 2012).

Gossypium, a close relative to *Hibiscus* in the Malvaceae family, is composed of diploids and allopolyploids. A study of 5S and 45S rDNA revealed that most diploids had two 5S rDNA signals and all allotetraploid species had four 5S rDNA signals (Gan et al., 2013), compared with only two 5S rDNA signals in tetraploid *H. syriacus*. In addition to among-ploidy level variation in rDNA signals, it is possible to have variation within the same ploidy for a species. In a comparative analysis of species in Brassicaceae, species with the same chromosome number were found to have up to a 5-fold difference in rDNA sites (Hasterok et al., 2006). As only one tetraploid of *H.*

syriacus was investigated, the background variability in copy number of tetraploid rDNA signals remains to be discovered.

Low copy numbers of 5S rDNA signals are not unique to *H. syriacus*. Elimination of 5S rDNA after polyploidization has been studied in other woody plants such as *Rubus* (Wang et al., 2015b). Both diploid and tetraploid species of *Rubus* carried two 5S sites where triploid and octoploid carried only three (Wang et al., 2015b). This phenomenon is common in FISH studies within the Rosaceae as reported for *Fragaria* (Liu and Davis, 2011), *Prunus* (Maghuly et al., 2010), and *Sanguisorba* (Mishima et al., 2002). However, to our knowledge this is the first report of possible 5S rDNA site elimination in *Hibiscus*.

Elimination of 5S rDNA sites in *H. syriacus* could lend evidence to its possible allopolyploid origin. Loss of rDNA has been studied in other allopolyploid taxa such as *Gossypium* (Wendel et al., 1995), *Nicotiana tabacum* (Volkov et al., 1999), *Tragopogon* (Kovarik et al., 2005), *Cardamine* (Franzke and Mummenhoff, 1999), and *Triticum* (Baum and Feldman, 2010). Drastic genome reorganization and modification often occur in newly formed allopolyploids (Kotseruba et al., 2003). Genes (rDNA) in these allopolypoids likely undergo the process of concerted evolution and interlocus homogenization (Álvarez and Wendel, 2003). These processes can result in significant changes in rDNA sites, including locus loss as demonstrated in the allotetraploid grass *Zingeria trichopoda* (Kotseruba et al., 2003). Evolution in allopolyploids often results in copy number and transcription changes in rDNA sites through a process of nucleolar dominance first described by Navashin (1934), where rDNA of one parent can be functionally dominant to the other parent in allopolyploids (Pikaard, 2000). Partial or whole copy loss of rDNA sites in allopolyploids also has occurred from suppressed/inactive nucleolar organizer regions (NORs) that prevent normal replication, leading to stepwise elimination of

rDNA as observed in cereals (Dvorak, 1990; Gustafson et al., 1988) and *N. tabacum* (Volkov et al., 1999). In addition, elimination of rDNA can occur rapidly in newly formed allopolyploids. Baum and Feldman (2010) found that in *Triticum*, elimination of 5S rDNA occurred within the first three generations after the formation of allopolyploids.

In the current study, putative pentaploids varied in 5S and 45S copy number. After interploid hybridization, putative pentaploid seedlings were selected for FISH analysis by flow cytometry of single leaf samples, which produced a range of intermediate genome sizes from 5.60 to 6.31 pg. (Table 2). To our knowledge, these selections represent the first FISH analysis on pentaploids from an interploid cross in *Hibiscus*. Studies on species with natural ploidy series, such as 4*x*, 5*x*, and 6*x* cytotypes of *Cenchrus ciliaris* (Kharrat-Souissi et al., 2012), have shown proportional increases of rDNA signals associated with each ploidy level. In contrast, a nonrandom distribution of interploid hybrid chromosomes and rDNA sites have been demonstrated in interspecific triploid hybrids of *Epidendrum*, where all triploids exhibited the same number of 5S and 45S signals as the diploid parent (Moraes et al., 2013). However, segregation patterns of rDNA signals in *H. syriacus* appeared to be random among the putative pentaploids and signals for each rDNA site ranged between the values of the two parents (Table 2).

From the 12 taxa investigated, all possible parental combinations of rDNA signals were found (Fig. 5). No obvious pattern was observed between parental combinations or genome size estimates and number of rDNA signals (Table 2). From the 12 taxa investigated, two groups of full-sib seedlings were evaluated. From the cross 'Aphrodite' × 'Pink Giant', three seedlings exhibited two combinations of rDNA signals: (H2013-017-11 = two 5S + four 45S) and [H2013-017-21, H2013-077-05 (reciprocal) = three 5S + five 45S]. From the cross 'Helene' × 'Pink Giant', two seedlings exhibited two combinations of rDNA signals: (H2013-124-19 = two 5S + five 45S) and (H2013-124-03 = three 5S + four 45S).

The role of polyploidy on plant morphology and physiology has been studied mostly in natural allopolyploids that have extinct diploid progenitors. The consequences of long spans of evolution following ancient hybridization and whole-genome duplication can obscure the role of polyploidy in variable morphology and physiology (Soltis et al., 2016). The development of vigorous taxa representing an interrelated, wide ploidy series in *H. syriacus* could prove useful in studying the effects of polyploidy on temperate shrubs. Future efforts with this ploidy series include producing clonal replicates of each taxon in the ploidy series and use of mature plants for measurements of morphological, anatomical, and physiological characteristics such as photosynthetic rate, drought tolerance, cold tolerance, pollen diameter, and fertility. Similar research has recently been performed on ploidy series in *Brassica* (Baker et al., 2017) and *A. thaliana* (Del Pozo and Ramirez-Parra, 2014). However, our ploidy series contains more cytotypes than the few studies on polyploidy and abiotic stress response in woody plants, such as *Lonicera japonica* (Li et al., 2009) and *Prunus salicina* (Pustovoitova et al., 1996). Woody, deciduous shrubs with a wide range of ploidy provide a unique opportunity to study winter cold tolerance in relation to ploidy, a topic rarely studied in current literature.

In this study, we identified genome size and ploidy variation in cultivars of *H. syriacus*. We successfully created a ploidy series consisting of 4*x*, 5*x*, 6*x*, and 8*x* cytotypes using a combination of interploid hybridization and autopolyploid induction. Among taxa in this ploidy series, we recorded variation in stomatal guard cell length, stomatal density, and copy number of fluorescent rDNA signals. In addition, we found random segregation of rDNA signals in a subset of pentaploid seedlings. This study will provide new opportunities for breeding novel cultivars of *H. syriacus* and the wide ploidy series will provide a foundation for studying physiological differences among ploidy levels in woody plants.

Literature Cited

Álvarez, I. and J.F. Wendel. 2003. Ribosomal ITS sequences and plant phylogenetic inferences. Mol. Phylogenet. Evol. 29:417–434.

Anamthawat-Jónsson, K. 2003. Preparation of chromosomes from plant leaf meristems for karyotype analysis and in situ hybridization. Methods Cell Sci. 25:91–95.

Baker, R.L., Y. Yarkhunova, K. Vidal, B.E. Ewers, and C. Weinig. 2017. Polyploidy and the relationship between leaf structure and function: Implications for correlated evolution of anatomy, morphology, and physiology in *Brassica*. BMC Plant Biol. 17:3, doi: 10.1186/s12870-016-0957-3.

Baum, B.R. and M. Feldman. 2010. Elimination of 5S DNA unit classes in newly formed allopolyploids of the genera *Aegilops* and *Triticum*. Genome 53:430–438.

Beaulieu, J.M., I.J. Leitch, S. Patel, A. Pendharkar, and C.A. Knight. 2008. Genome size is a strong predictor of cell size and stomatal density in angiosperms. New Phytol. 179:975–986.

Buechler, W.K. 2000. Estimating polyploidy levels using cell size in *Salix* leaves. Amer. J. Bot. 87:80–81.

Chang, Y.-C., C.-T. Shii, and M.C. Chung. 2009. Variations in ribosomal RNA gene loci in spider lily (*Lycoris* spp.). J. Amer. Soc. Hort. Sci. 134:567–573.

Chen, G., W.-B. Sun, and H. Sun. 2009. Morphological characteristics of leaf epidermis and size variation of leaf, flower and fruit in different ploidy levels in *Buddleja macrostachya* (Buddlejaceae). J. Syst. Evol. 47:231–236.

Chen, H., M.-C. Chung, Y.-C. Tsai, F.-J. Wei, J.-S. Hsieh, and Y.-I.C. Hsing. 2015. Distribution of new satellites and simple sequence repeats in annual and perennial *Glycine* species. Bot. Stud. 56:22.

Chung, M.-C., Y.-I. Lee, Y.-Y. Cheng, Y.-J. Chou, and C.-F. Lu. 2008. Chromosomal polymorphism of ribosomal genes in the genus *Oryza*. Theor. Appl. Genet. 116:745–753.

Contreras, R. and J.D. Lattier. 2014. Improving a garden classic. Digger 2014(Aug):47–50.

Contreras, R.N., J.M. Ruter, and W.W. Hanna. 2009. An oryzalin-induced autoallooctoploid of *Hibiscus acetosella* Welw. Ex. Hiern. 'Panama Red' (Malvaceae). J. Amer. Soc. Hort. Sci. 134:553–559.

Del Pozo, J.C. and E. Ramirez-Parra. 2014. Deciphering the molecular bases for drought tolerance in *Arabidopsis* autotetraploids. Plant Cell Environ. 37:2722–2737.

Dvorak, J. 1990. Evolution of multigene families: The ribosomal RNA loci of wheat and related species, p. 83–97. In: A.H.D. Brown, M.T. Clegg, A.L. Kahler, and B.S. Weir (eds.). Plant population genetics, breeding, and genetic resources. Sinauer Assoc., Sunderland, MA.

Eeckhaut, T.G.R., J.M. Van Huylenbroeck, J. De Riek, and E. Van Bockstaele. 2004. Interspecific hybridization between *Hibiscus syriacus* L. and *Hibiscus paramutabilis* Bailey. Acta Hort. 630:85–90.

Egolf, D.R. 1970. *Hibiscus syriacus* 'Diana', a new cultivar. Baileya 17:75–78.

Egolf, D.R. 1981. 'Helene' rose of sharon (althea). HortScience 16:226–227.

Egolf, D.R. 1986. 'Minerva' rose of sharon (althea). HortScience 21:1463–1464.

Egolf, D.R. 1988. 'Aphrodite' rose of sharon (althea). HortScience 23:223.

Essiett, U.A. and E.S. Iwok. 2014. Floral and leaf anatomy of *Hibiscus* species. Amer. J. Medical Biol. Res. 2:101–117.

138

J. Amer. Soc. Hort. Sci. 144(2):130–140. 2019.

Fantz, P.R. 1994. Taxonomic problems in cultivated liriopogons. HortTechnology 3:146–150.

Franzke, A. and K. Mummenhoff. 1999. Recent hybrid speciation in *Cardamine* (Brassicaceae)—Conversion of nuclear ribosomal ITS sequences in statu nascendi. Theor. Appl. Genet. 98:831–834.

Fryxell, P.A. 1988. Malvaceae of Mexico. Syst. Bot. Monogr. 25:1–522.

Gan, Y., F. Liu, D. Chen, Q. Wu, Q. Qin, C. Wang, S. Li, X. Zhang, Y. Wang, and K. Wang. 2013. Chromosomal locations of 5S and 45S rDNA in *Gossypium* genus and its phylogenetic implications revealed by FISH. PLoS One 8:e68207, doi: 10.1371/journal.pone.0068207.

Gerlach, W.L. and J.R. Bedbrook. 1979. Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res. 7:1869–1885.

Gerlach, W.L. and T.A. Dyer. 1980. Sequence organization of the repeated units in the nucleus of wheat which contain 5S rRNA genes. Nucleic Acids Res. 8:4851–4865.

Gomes, S.S.L., C.W. Saldanha, C.S. Neves, M. Trevizani, N.R.B. Raposa, M.M. Notini, M.O. Santos, J.M.S. Campos, W.C. Otoni, and L.F. Viccini. 2014. Karyotype, genome size, and in vitro chromosome doubling of *Pfaffia glomerata* (Spreng.) Pedersen. Plant Cell Tissue Organ Cult. 118:45–56.

Greilhuber, J., J. Doležel, M.A. Lysák, and M.D. Bennett. 2005. The origin, evolution and proposed stabilization of the terms 'genome size' and 'C-value' to describe nuclear DNA contents. Ann. Bot. 95:255–260.

Gustafson, J.P., A.R. Dera, and S. Petrovic. 1988. Expression of modified rye ribosomal RNA genes in wheat. Proc. Natl. Acad. Sci. USA 85:3943–3945.

Hasterok, R., E. Wolny, M. Hosiawa, M. Kowalczyk, S. Kulak-Ksiazczyk, T. Ksiazczyk, W.K. Heneen, and J. Maluszynska. 2006. Comparative analysis of rDNA distribution in chromosomes of various species of Brassicaceae. Ann. Bot. 97:205–216.

Joly, S. and A. Bruneau. 2007. Delimiting species boundaries in *Rosa* Sect. Cinnamomeae (Rosaceae) in eastern North America. Syst. Bot. 32:819–836.

Kharrat-Souissi, A., S. Siljak-Yakovlev, F. Pustahija, and M. Chaieb. 2012. Physical mapping of 5S and 18S-5.8S-26S RNA gene families in polyploid series of *Cenchrus ciliaris* Linnaeus, 1771 (Poaceae). Comp. Cytogenet. 6:273–286.

Kim, Y.-M., S. Kim, N. Koo, A.-Y. Shin, S.-I. Yeom, E. Seo, S.-J. Park, W.-H. Kang, M.-S. Kim, J. Park, I. Jang, P.-G. Kim, I. Byeon, M.-S. Kim, J. Choi, G. Ko, J. Hwang, T.-J. Yang, S.-B. Choi, J.M. Lee, K.-B. Lim, J. Lee, I.-Y. Choi, B.-S. Park, S.-Y. Kwon, D. Choi, and R.W. Kim. 2017. Genome analysis of *Hibiscus syriacus* insights of polyploidization and indeterminate flowering in woody plants. DNA Res. 24:71–80.

Kotseruba, V., D. Gernand, A. Meister, and A. Houben. 2003. Uniparental loss of ribosomal DNA in the allotetraploid grass *Zingeria trichopoda* (2*n* = 8). Genome 46:156–163.

Kovarik, A., J.C. Pires, A.R. Leitch, K.Y. Lim, A.M. Sherwood, R. Matyasek, J. Rocca, D.E. Soltis, and P.S. Soltis. 2005. Rapid concerted evolution of nuclear ribosomal DNA in two *Tragopogon* allopolyploids of recent and recurrent origin. Genetics 169:931–944.

Lattier, J.D., H. Chen, and R.N. Contreras. 2017. Improved method of enzyme digestion for root tip cytology. HortScience 52:1029–1032.

Lattier, J.D., T.G. Ranney, and N.P. Lynch. 2013. History and cytological reassessment of *Rhododendron canadense*. J. Amer. Rhododendr. Soc. 67:92–98.

Lee, S.K. and C.S. Kim. 1976. Studies on artificial polyploidy forest trees XIII: Some morphological and physiological characteristics of colchitetraploid *Hibiscus syriacus* L. J. Korean For. Soc. 32:73–86.

Li, W.-D., D.K. Biswas, H. Xu, C.-Q. Xu, X.-Z. Wang, J.-K. Liu, and G.-M. Jiang. 2009. Photosynthetic responses to chromosome doubling in relation to leaf anatomy in *Lonicera japonica* subjected to water stress. Funct. Plant Biol. 36:783–792.

Li, W.L., G.P. Berlyn, and P.M.S. Ashton. 1996. Polyploids and their structural and physiological characteristics relative to water deficit in *Betula papyrifera* (Betulaceae). Amer. J. Bot. 83:15–20.

Li, Z. and J.M. Ruter. 2017. Development and evaluation of diploid and polyploid *Hibiscus moscheutos*. HortScience 52:676–681.

Liu, B. and T.M. Davis. 2011. Conservation and loss of ribosomal RNA gene sites in diploid and polyploid *Fragaria* (Rosaceae). BMC Plant Biol. 11:157.

Maghuly, F., B. Schmoellorl, E.M. Temsch, and M. Laimer. 2010. Genome size, karyotyping and FISH physical mapping of 45S and 5S genes in two cherry rootstocks: *Prunus subhirtella* and *Prunus incisa* × *serrula*. J. Biotechnol. 149:88–94.

McGoey, B.V., K. Chau, and T.A. Dickenson. 2014. Stomata size in relation to ploidy level in North American hawthorns (*Crataegus*, Rosaceae). Madrono 61:177–193.

Menzel, M.Y. and E.D. Wilson. 1969. Genetic relationships in *Hibiscus* sect. *Furcaria*. Brittonia 21:91–125.

Mishima, M., N. Ohmido, K. Fukui, and T. Yahara. 2002. Trends in site-number change of rDNA loci during polyploid evolution in *Sanguisorba* (Rosaceae). Chromosoma 110:550–558.

Moraes, A.P., M. Chinaglia, C. Palma-Silva, and F. Pinheiro. 2013. Interploidy hybridization in sympatric zones: The formation of *Epidendrum fulgens* × *E. puniceoluteum* hybrids (Epidendroideae, Orchidaceae). Ecol. Evol. 3:3824–3837.

Navashin, M. 1934. Chromosomal alterations caused by hybridization and their bearing upon certain general genetic problems. Cytologia 5:169–203.

Ochatt, S.J. 2008. Flow cytometry in plant breeding. Cytometry 73:581–598.

Padoan, D., A. Mossad, B. Chiancone, M.A. Germana, and P.S.S.V. Khan. 2013. Ploidy levels in *Citrus clementine* affects leaf morphology, stomatal density and water content. Theor. Exp. Plant Physiol. 25:283–290.

Pikaard, G.S. 2000. The epigenetics of nucleolar dominance. Trends Genet. 16:495–500.

Prado, E.P., P. Faivre-Rampant, C. Schneider, and M.A. Darmency. 1996. Detection of a variable number of ribosomal DNA loci by fluorescent in situ hybridization in *Populus* species. Genome 39:1020–1026.

Pustovoitova, T.N., G.V. Eremin, E.G. Rassvetaeva, and V.N. Zholkevich. 1996. Drought resistance, recovery capacity, and phytohormone content in polyploid plum leaves. Russ. J. Plant Physiol. 43:232–235.

Ribeiro, T., A. Barão, W. Viegas, and L. Morais-Cecílio. 2008. Molecular cytogenetics of forest trees. Cytogenet. Genome Res. 120:220–227.

Saltonstall, K., K. Glennon, A. Burnett, R.B. Hunter, and K.L. Hunter. 2007. Comparison of morphological variation indicative of ploidy level in *Phragmites australis* (Poaceae) from eastern North America. Rhodora 109:415–429.

Satya, P., M. Karan, D. Sarkar, and M.K. Sinha. 2012. Genome synteny and evolution of AABB allotetraploids in *Hibiscus* section *Furcaria* revealed by interspecific hybridization, ISSR and SSR markers. Plant Syst. Evol. 298:1257–1270.

Shim, K.K., K.H. Kim, and Y.M. Ha. 1993. Characteristics of triploid cultivars 'Diana' and 'Helene' in *Hibiscus syriacus* L. J. Korean Soc. Hort. Sci. 34:54–67.

Skovsted, A. 1941. Chromosome numbers in the Malvaceae II. Comptes rendus des traveaux du laboratoire Carlberg. Série Physiologique 23:195–242.

Soltis, D.E., C.J. Visger, D.B. Marchant, and P.S. Soltis. 2016. Polyploidy: Pitfalls and paths to a paradigm. Amer. J. Bot. 103:1–21.

Srivastava, A.K. and D. Schlessinger. 1991. Structure and organization of ribosomal DNA. Biochimie 73:631–638.

Van Huylenbroeck, J.M., J. de Riek, and M. De Loose. 2000. Genetic relationships among *Hibiscus syriacus, Hibiscus sinosyriacus* and *Hibiscus paramutabilis* revealed by AFLP, morphology and ploidy analysis. Genet. Resources Crop Evol. 47:335–343.

Van Laere, K., A. Dewitte, J. Van Huylenbroeck, and E. Van Bockstaele. 2009. Evidence for the occurrence of unreduced gametes

J. Amer. Soc. Hort. Sci. 144(2):130–140. 2019.

139

in interspecific hybrids of *Hibiscus*. J. Hort. Sci. Biotechnol. 84:240–247.

Van Laere, K., J. Van Huylenbroeck, and E. Van Bockstaele. 2006. Breeding strategies for genetic variability within *Hibiscus syriacus*. Acta Hort. 714:75–81.

Van Laere, K., J.M. Van Huylenbroeck, and E. Van Bockstaele. 2007. Interspecific hybridization between *Hibiscus syriacus, Hibiscus sinosyriacus*, and *Hibiscus paramutabilis*. Euphytica 155:271–283.

Volkov, R.A., N.V. Borisjuk, I.I. Panchuk, D. Schweizer, and V. Hemleben. 1999. Elimination and rearrangement of parental rDNA in the allotetraploid *Nicotiana tobacum*. Mol. Biol. Evol. 16:311–320.

Volkov, R.A., I.I. Panchuk, N.V. Borisjuk, M. Hosiawa-Baranska, J. Maluszynska, and V. Hemleben. 2017. Evolutional dynamics of 45S and 5S ribosomal DNA in ancient allohexaploid *Atropa belladonna*. BMC Plant Biol. 17:21, doi: 10.1186/s12870-017-0978-6.

Wang, Q., J. Wang, Y. Zhang, Y. Zhang, S. Xu, and Y. Lu. 2015a. The application of fluorescent in situ hybridization in different ploidy levels cross-breeding of lily. PLoS One 10:e0126899, doi: 10.1371/journal.pone.0126899.

Wang, Y., X. Wang, Q. Chen, L. Zhang, H. Tang, Y. Luo, and Z. Liu. 2015b. Phylogenetic insights into subgenera *Idaeobatus* and *Malachobatus* (*Rubus*, Rosaceae) inferring from ISH analysis. Mol. Cytogenet. 8:11, doi: 10.1186/s13039-015-0114-y.

Wendel, J.F., A. Schnabel, and T. Seelanan. 1995. Bidirectional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). Proc. Natl. Acad. Sci. USA 92:280–284.

Wilson, E.D. 1994. The genome biogeography of *Hibiscus* L. section *Furcaria* DC. Genet. Resources Crop Evol. 41:13–25.

Wilson, E.D. 1999. Revision of *Hibiscus* section *Furcaria* (Malvaceae) in Africa and Asia. Bul. Natl. History Museum London Bot. 29:47–79.

Xiong, Y.-C., F.-M. Li, and T. Zhang. 2006. Performance of wheat crops with different chromosome ploidy: Root-sourced signals, drought tolerance, and yield performance. Planta 224:710–718.

Zhuang, D.-H. and J.-J. Song. 2005. The characters of pollen grains and stomatal apparatus in *Hibiscus* L. in relation to ploidy. J. Trop. Subtrop. Bot. 13:49–52.

140

J. Amer. Soc. Hort. Sci. 144(2):130–140. 2019.