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Development of triploid daylily (*Hemerocallis*) germplasm by embryo rescue

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Abstract Interploidy crosses between diploid daylily (*Hemerocallis*) cultivars (2n = 22) and tetraploid cultivars (2n = 44) were performed via in vivo hybridization aiming to produce triploid hybrid germplasm (3n = 33). Plant growth regulator-free MS based medium containing 5 levels of sucrose—1, 2, 3, 4 or 5% was used to optimize the embryo rescue medium. It was determined that the medium supplemented with 3% sucrose gave highest rate of immature hybrid embryo rescue. Thirty-seven hypothetically triploid genotypes were subjected to verification of ploidy status by root tip chromosome counting and flow cytometry. Thirty-one of them were confirmed triploids. These genotypes were in vitro propagated, acclimatized to ex vitro conditions, and planted for future field performance evaluation and environmental testing. The newly developed triploid genotypes could open new horizons for further polyploidy breeding.

Keywords Polyploidy · Cytology · Flow cytometry · Micropropagation · Tissue culture

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Introduction

Daylily (*Hemerocallis* spp., *Liliaceae*) is one of the most economically important flowering perennial nursery crops in the United States because of its large, showy flowers and its adaptation to a wide range of soil and climates (Munson 1989). There are 64,329 daylilies registered by the American Hemerocallis Society (AHS 2009). In spite of the increased number of registered cultivars per annum, the genetic diversity of daylily cultivars is rapidly decreasing (Tomkins et al. 2001). This trend continues to develop because current breeding efforts target developing predominantly tetraploid daylily cultivars by chromosome doubling of a limited number of diploid genotypes, and further cross hybridization between the resulting even fewer tetraploids with unique characteristics (Sakhanokho et al. 2004). Therefore, expanding the genetic diversity in *Hemerocallis* spp. is necessary, and triploid breeding provides the unique opportunity to improve daylily's biodiversity, performance, and its market value in a fast and efficient way (Brennan 1992).

In nature, daylily species are often diploids with 22 chromosomes (Brennan 1992; Plodeck 2002; Stout 1934). Tetraploid (4n = 44) daylilies have also been developed by chromosome doubling of diploid plants through chemical treatments, such as colchicine (Tomkins et al. 2001). Tetraploids usually have larger flowers and larger plants compared to diploids, and are increasingly popular among hybridizers and within the perennial nursery market. Although occurrence of triploid (3n = 33) daylilies due to spontaneous mutations have been reported (Matsuoka 1971), such germplasm is rare in nature and has very localized distribution because normally it will be reproductively sterile. The triploid *H. fulva* cv. Kwanso does not develop female flower parts at all (Dark 1932). Triploid plant cells contain three non-homologous chromosomes sets, which will not complete meiosis (Bennett 2004), In fact, triploid daylily embryos can be achieved by crossing diploid and tetraploid genotypes; however, they are poorly developed and as a result about 50% of the pods abort in a week after pollination, and others abscise at various stages of their maturity during the following weeks (Arisumi 1973). Therefore, it is nearly impossible to develop triploid daylilies by traditional cross breeding only (Arisumi 1970), and the need of developing embryo rescue protocol is evident. In addition, development of a highly efficient micropropagation protocol would allow maintaining the triploid population in vitro, and more importantly—would enable commercial scale propagation.

Historically the development of polyploids proved to be a very useful and valuable tool to improve traits in plant breeding (Bennett 2004; Notzuka et al. 2000). Both—long lasting blooming and especially re-blooming of daylily are among the most desirable characteristics sought for public and home gardens, parks, road-side plantings, and for professional and hobby hybridizing programs. Due to the uneven distribution of genetic material triploid daylily germplasm should not normally set seeds, and more nutrients will be directed to flower development. Therefore, we hypothesize that the sterility in triploid daylily germplasm should result in improved tendency for continuous blooming and/or more distinctive re-blooming pattern even in regions with cooler climates where the latter characteristic is rarely exhibited. The initial results from our field tests, although yet insufficient for reporting, indicate possibility for such trend.

Triploid daylilies are also a very good starting material for hexaploid breeding by chromosome doubling techniques (Morejohn et al. 1987). According to Levin (1983) and Horn (2002) development of hexaploid daylily may increase the size of flowers, intensify the flower colors, modify plant shape and restore fertility from triploids. Creating hexaploid daylily germplasm would greatly increase the opportunities for further germplasm improvement by traditional hybridizers.

Here we report the development of a complete methodology for breeding and propagation of triploid daylily germplasm. Protocols for hybrid embryo rescue, in vitro propagation of triploid daylily, and a method for characterization of triploid daylily germplasm were successfully established. Thirty-one triploid genotypes have been developed in 2005 and 2006, multiplied, acclimatized, and planted for field trials.

Materials and methods

Parental germplasm and hybridization

Sixty diploid and twenty-five tetraploid daylily cultivars with excellent ornamental value and tendency to re-bloom in Virginia were chosen as parental plants. Manual pollination was performed in order to guarantee the genetic status of the triploid hybrid seeds from crosses between desired tetraploid × diploid, or diploid × tetraploid parents. Capsules with the immature seeds resulting from the successful controlled hybridizations were collected 10–12 days after pollination (DAP) for embryo rescue.

Hybrid embryo rescue and plantlet micropropagation

The capsules (10–12 DAP) of each cross-combination were rinsed with tap water, surface disinfected in 70% (v/v) ethanol for 1 minute, then in 30% (v/v) commercial bleach (5.25% sodium hypochlorite) for 20 min, followed by four rinses in sterile, deionized water. Capsules were aseptically opened to remove ovules. Immature seeds were germinated at 25°C on growth regulators-free MS medium (Murashige and Skoog 1962) supplemented with one of five sucrose

concentrations (1, 2, 3, 4, or 5%) and 0.7% agar (pH.5.8). For each experiment, five Petri dishes were used for each medium test. Each dish contained about 20 hybrid embryos. The experiment was repeated three times as three replications. The germination data was analyzed using Duncan's new multiple range tests. Six weeks after culture initiation under darkness, all seeds with roots were sub-cultured on same media and transferred to 16 h/8 h light/dark regime photoperiod using cool, white fluorescence light of 40 μmolm^{-2} s^{-1} which was around 25 cm above the Petri dishes. After 4–6 weeks, germinated plantlets were transferred onto propagation magenta boxes containing growth regulators-free ½ strength MS medium containing ½ MS basal salts and vitamins, 2% (w/v) sucrose and 0.7% (w/v) agar, pH 5.8. Plantlets were then transferred every 4 weeks for propagation to fresh ½ strength MS medium without growth regulators. Plantlets in two-leaf stage were planted in 10 cm diameter plastic pots containing sterile soilless medium and covered by a plastic bag to provide high relative humidity. Plantlets were acclimatized to ex vitro conditions by gradually opening the bag during 4–6 weeks in growth chamber at 25°C with 16 h/8 h light/dark regime photoperiod with fluorescence light of 450 μmol m^{-2} s^{-1} around 20 cm above the plantlets.

Verification of ploidy status

Flow cytometry analysis

Basal tissues of young and healthy leaves of putative triploid daylilies were used for flow cytometry analysis. Young and healthy leaves of 7 known diploid and 4 tetraploid daylily cultivars, which were parents of the presumably triploid daylilies, were used as standards. Plain-leaf parsley (*Petroselinum crispum* (Mill) Nym. ex A.W. Hill) was also used as a control, because the DNA content of parsley is found to be exactly half of that of a diploid daylily genotype (Saito et al. 2003). The relative fluorescence intensity (RFI) ratio of nuclei from each daylily sample and parsley sample was used to determine the ploidy level of each daylily genotype. Leaf tissue samples—about 0.5 cm × 0.5 cm, were chopped using a sharp razor for 30–60 s into 0.5 ml extraction buffer (CyStain P1 Absolute P, Partec GmbH, Munster, Germany). After 60–90 s of incubation in the extraction buffer each sample was filtered through a Partec 50 CellTrics filter (Partec GmbH, Munster, Germany). The filtered samples were then stained with 1.0 ml staining solution (with 0.6% P1 solution and 0.02 mg RNAse) (Partec GmbH, Munster, Germany) in a test tube for one hour and the fluorescence intensity was read in the red channel using a flow cytometer (Coulter EPICS XL-MCL, Miami, FL).

Chromosome count

Chromosome numbers were determined in meristematic cells of root tips taken from potted plants maintained in the growth chamber. Root tips (1–2 cm) were pre-treated in a saturated solution of 1-bromonaphthalene for 2 h, and fixed in 3:1 (alcohol:glacial acetic acid) for 24 h. They were then hydrolyzed in 1 N HCl at 60°C for 10 min, and transferred to a 2% pectinase solution for 45 min at room temperature. Root tips were then stained with 0.5% aceto-carmine. The overstained cytoplasm was destained by applying a drop of a mixture of 1 part 1% acetocarmine and 1 part 1 N HC1. To prepare a squash, 1 mm of the tip of the stained root was cut off, placed on a slide, and a drop of 45% acetic acid and glycerin (10:1) was added. The root tip was covered with a plastic cover slip and tapped several times with a dull needle to break up the tip and disperse the cells. The slide was placed between two pieces of folded filter paper and moderate thumb pressure was applied to flatten the cells. The chromosome number was counted under light microscope (Olympus-BX51TRF, Tokyo, Japan).

Results and discussion

Although some attempts for triploid breeding were made as early as 1970s (Arisumi 1970, 1973), no progress has been made since then, resulting in no triploid hybrids being commercially available. The critical procedures for successful triploid breeding are hybrid embryo rescue, micro-propagation and verification of the desired ploidy level. Here, we report the successful development of a system for efficient development of triploid daylily germplasm by combined application of traditional and in vitro approaches.

Development of a high efficient medium for embryo rescue

Most of cross-pollinations resulted in seed capsules; however around 85% of the harvested pods contained some seeds (embryos) 12 days after the pollination. Onset of seed abortion could be observed within 10–20 days after a cross was made between diploid and tetraploid parents. Therefore, we harvested all the capsules around 10–12 DAP and started the embryo rescue. The potential role of maternal effect on survivability rate in daylily embryos is unclear as no literature data whether the direction of a cross effects the quantity of vital triploid embryos in daylily is available. Arisumi reports that he made 1,085 crosses between diploid and tetraploid cvs, and 522 between tetraploid and diploid ones, however the author does not comment if the direction of crosses had any effect on the number of seeds obtained (Arisumi 1973). We also performed straight (2n × 4n) and reversed (4n × 2n) crosses with the same parents and monitored the outcomes. We found no significant difference in seed set (data not shown), however the potential maternal effect on seed set need to be further studied. Once capsules were harvested and hybrid seeds placed on standard MS medium with 2% sucrose, pH 5.8, most of the embryos turned brown, wilted within 3–5 days and then died. Some of the embryos which survived on the rescue medium produced first roots in the dark within 2–3 weeks and then germinated shoots under light within 1–2 weeks.

After experimenting to optimize several components of the medium (data not shown), we found that the sucrose content of the MS medium is the most important factor for the survival rates of hybrid embryos (Fig. 1). The medium containing 3% sucrose resulted in a 3.17% embryo rescue rate, which was significantly higher than the other treatments. The sucrose concentration below or above 3% greatly decreased the germination percentage. This result indicates that sucrose is not only an important carbon source, but also a critical component for in vitro embryo germination. The different levels of embryo germination in relation to sucrose concentration are most likely due to the fact that sucrose affects the germination by adjusting the osmotic pressure of the medium (Raghavan 2003).

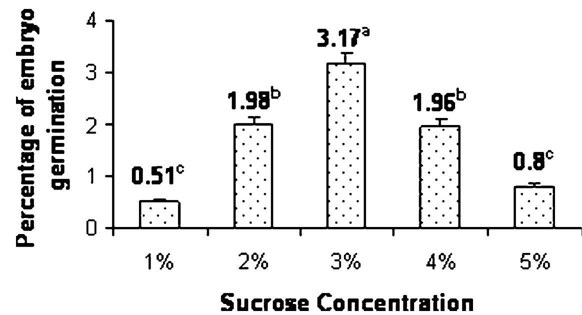

Fig. 1 The effect of sucrose concentration in embryo rescue medium on the embryo germination rates. Error bars indicate standard errors from mean values. Letters above the plots indicate significance of differences between mean values ($P = 0.05$). The same letter shows no significant difference, and different letters show significant difference

In vitro propagation

A total of 37 genotypes of putative triploid embryos were successfully germinated. The rescued embryos matured and developed into plantlets within 40–60 days on the optimized embryo rescue medium. The hybrid plantlets were then transferred on propagation media to produce clusters of multiple shoots. The plantlets start growing vigorously on the growth regulator-free propagation medium one week after the transfer. Each plantlet could produce 2–5 multiple shoot clusters within 4–6 weeks on the propagation medium. Four- to six-weeks old clusters of shoots were then separated and all of them successfully produced roots on the ½ MS medium without growth regulator. Our choice to eliminate growth regulators from medium supplements was dictated by the fact that their use is often associated with induction of genetic and phenotypic variations, which is unacceptable from nursery industry in cases where “trueness to type” is imperative. The micro-propagation method we used provides for a practical way to scale-up triploid daylily germplasm for potential commercial development.

Verification of triploids by flow cytometry and chromosome counting

Flow cytometry has frequently been used in plant ploidy analysis (Brummer et al. 1999; Costich et al.

1993; Dansi et al. 2001). Flow cytometry analysis of leaf nuclei is an efficient way to measure DNA content and determine ploidy of daylily (Saito et al. 2003). Flow cytometry analysis of daylily and parsley samples, with known ploidy levels, showed that although there was some variation within a ploidy level, the distribution of RFI is discontinuous with distinct range between different ploidy levels (Fig. 2). Therefore, the ploidy levels can be easily inferred. The FI for parsley fell in the haploid range, nearly half the FI of the diploids. Estimated DNA content of triploids and tetraploids by flow cytometry were approximately three times and four times of that of the parsley, respectively. In this report, 37 putative triploids, as well as 7 known diploids and 4 known tetraploids, were analyzed by flow cytometry. From the RFI frequency distribution plot (Fig. 3), the RFI value for these 48 tested samples could be easily classified into three groups: 11 genotypes were classified as diploids with RFI range from 1.86 to 2.38 (group A), 31 genotypes were determined as triploids with RFI range 2.88–3.48 (group B), and 6 genotypes were found to be tetraploids with RFI range 3.82-4.24 (group C). As expected, the RFI of all known diploids and tetraploids used as standards fell exactly in the range of their ploidy groups, which proved the reliability of the method used. To further verify the results of flow cytometry analysis, 6 genotypes from the triploid group were randomly selected for root tip chromosome counting. The chromosome count was 33 in all of them (Fig. 4). This supports the previous finding that all the 31 genotypes identified as triploid through flow cytometry are true triploids. We have also found that 6 of the presumably triploid plants were actually not: four of them were classified as diploids, and two of them were classified as tetraploids. This most likely was due to failure of attempted interploidy cross (2n × 4n, or 4n × 2n) and the occurrence of self-pollination (2n × 2n, or 4n × 4n).

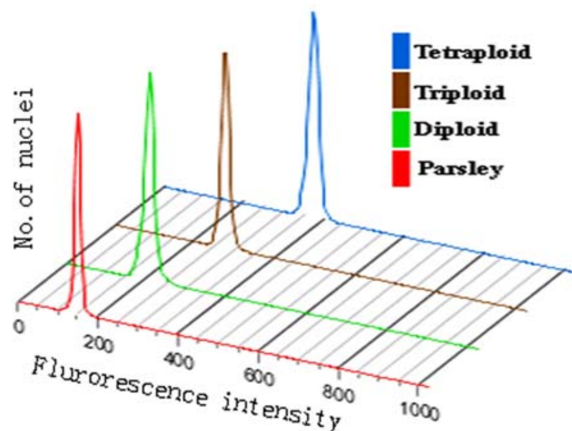

Fig. 2 Flow cytometry analyses show the relationship between the fluorescence intensity and the nuclear DNA content of *Hemerocallis* genotypes with different ploidy level (3D picture generated by the flow cytometer software). Mean values of fluorescence intensity of diploid, triploid and tetraploid samples were approximately two times, three times and four times of that of the parsley respectively

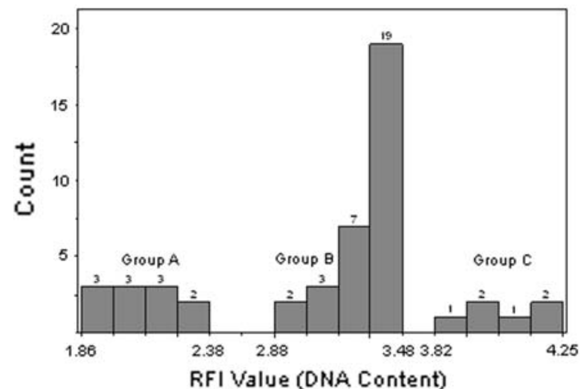

Fig. 3 Frequency distribution of the relative fluorescence intensity to parsley from each *Hemerocallis* leaf samples. The RFI (Fluorescence Intensity ratio of unknown sample to parsley) values were grouped into three distinct groups, which stood for different ploidy level of *Hemerocallis app*

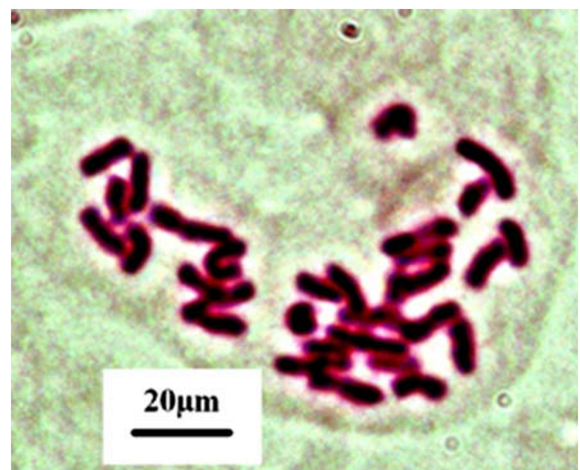

Fig. 4 Representative chromosome counting: analyzes of root tips of the putative triploids proved that the plants contain 3n = 33 chromosomes

This paper reports on establishing a complete, effective and repeatable set of procedures for triploid breeding, hybrid embryo rescue, and in vitro propagation. This comprehensive process enables production of triploid germplasm on a large scale, which is a prerequisite for successful selection of material with commercial potential. The first set of our triploids have been planted in field trials and their evaluation for horticultural performance, esthetic value, and market potential has begun.

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