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Efficient Ploidy Determination of Anther-derived Broccoli

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Abstract. **Broccoli (*Brassica oleracea* L. Italica Group) breeders routinely use anther or microspore culture to produce doubled-haploid (DH), homozygous lines. In addition to DH (diploid) regenerants, haploid, triploid, tetraploid, octaploid, and aneuploid regenerants may also result from anther culture. Thus, regenerated populations must be screened to identify the diploids, which are the only regenerants likely to set seed and serve as inbred lines. DNA flow cytometry is a useful procedure to determine ploidy of anther-derived regenerants. This study was undertaken to evaluate the effect of plant stage and sampling procedures on ploidy determination by flow cytometry. Anther-derived plants were analyzed at both seedling and mature plant stages. In separate tests, leaves were sampled on a given date, and stability of flow cytometry preparations were evaluated at 1, 2, 4, and 7 days after preparation. In addition, the stability of ploidy readings of excised leaves stored at 4 °C was examined over a 7-day period. In 139 out of 140 comparative assays there was no effect of plant stage on ploidy determination. Flow cytometry preparations stored at 4 °C gave consistent ploidy determinations up to 2 days after they were made, but some instability was observed by 4 and 7 days. Refrigerated leaves were more stable than nuclei preparations, and ploidy determinations did not differ from the first sampling through storage for 7 days. Results indicate that broccoli breeders could make flow cytometry preparations on site and send them offsite for flow cytometry analysis as long as analysis was completed within 1 or 2 days of sample preparation. More consistent results would be obtained by refrigerating leaves and sending them offsite for preparation and analysis at the offsite location.**

The production of doubled-haploid (DH) lines in *Brassica oleracea* L. using anther or microspore culture has become a breeding technique commonly employed by cole crop breeders (Duijs et al., 1992; Keller, 1984). Anther culture has been used widely for *B. oleracea* DH development (Chauvin et al., 1993; Keller and Armstrong, 1981; Ockendon, 1984, 1988); direct microspore culture has been employed more recently (Duijs et al., 1992). A complication in producing anther-derived DH plants in *B. oleracea* is that populations arising from culture contain a mixture of plants with different ploidy levels (Chauvin et al., 1993; Ockendon, 1986, 1988). In general, microspore-derived embryos undergo a polyploidization or relatively random doubling, tripling, quadrupling, or octupling of the entire genome. Thus, regenerated populations include haploids, diploids, triploids, tetraploids, and octaploids. Aneuploids are rare but they also have been observed among regenerants (Chauvin et al., 1993).

The mixture of ploidy levels of plants regenerated from anther culture presents the breeder with the difficult task of specifically identifying the diploids or true DH plants. The DH plants serve as potential homozygous lines to be used by the breeder in hybrid combinations, while plants with other ploidy levels have little practical value. Because only the DH plants are likely to set seed (Chauvin et al., 1993; Farnham, 1996), it is especially important that a breeder determines ploidy of regenerants and eliminates everything except the DHs before initiating expensive and time-consuming self-pollinations.

Cole crop breeders using anther culture attempt to maximize the numbers (i.e., hundreds) of plants in regenerated populations, and thus they need a straightforward and fast method for identifying diploid or DH plants to increase the overall efficiency of developing DH lines. From seedling stages through maturity, there are no obvious morphological characteristics that distinguish diploids from other ploidy types. At flowering, haploids can be distinguished from other types by the appearance of abnormally small flower buds (Keller, 1984). However, diploids and tetraploids appear similar even at flowering.

Ploidy determination by cytological evaluation of meiotic or mitotic stages has proven useful in evaluating small populations of anther-derived plants (Chiang et al., 1985), but is impractical for large populations. As an alternative, Ockendon (1986, 1988) found that guard cell length in the leaf epidermis is highly correlated with ploidy level in brussels sprouts (*B. oleracea* L. Gemmifera Group) and cauliflower (*B. oleracea* L. Botrytis Group). Using this correlated trait, he showed that anther culture of brussels sprouts resulted in populations of ≈50% haploids, 45% diploids, and 5% other ploidy levels (Ockendon, 1986), and that culture of cauliflower resulted in populations that were 1% haploid, 41% diploid, 5% triploid, and 53% tetraploid (Ockendon, 1988).

DNA flow cytometry has been used to estimate nuclear DNA content of many plant species (Arumaganathan and Earle, 1991a; Bennett and Leitch, 1995). Flow cytometry has also been used to differentiate ploidy of different individuals in variable populations of sugar beet (*Beta vulgaris* L.) by DeLaat et al. (1987). Similarly, ploidy of potato (*Solanum tuberosum* L.) genotypes has been determined using flow cytometry (Ramulu and Dijkhuis, 1986). Chauvin et al. (1993) determined ploidy of anther-derived broccoli and cauliflower using flow cytometry and observed populations similar to those of cauliflower described by Ockendon (1988). However, all samples were taken at one time prior to flowering, and Chauvin et al. (1993) gave few details of their cytometry techniques or any indication of how accurate their methods were. In tobacco (*Nicotiana tabacum* L.), leaf age can have a significant effect on characteristics of DNA histograms from flow cytometry (Galbraith et al., 1983). We are unaware of any studies with *B. oleracea* that have examined effects of plant or leaf age on results obtained with flow cytometry.

In this study, the overall goal was to better elucidate the use of flow cytometry for practical application to a breeding program developing DH broccoli. In particular, one objective was to determine the effect of sampling seedlings vs. mature plants on ploidy analysis by flow cytometry. A second objective was to evaluate the stability of flow cytometry preparations over time, and also to determine if whole leaves can be refrigerated, stored, and subsequently used for flow cytometry analysis. If flow cytometry preparations from leaves or stored leaves remain stable for several days before analysis, this technique could be used in a breeding program where preparations are analyzed offsite. A final objective of this research was to measure DNA content of haploid, diploid, triploid, and tetraploid individuals.

Materials and Methods

Anther culture and production of regenerants. All phases of the anther culture process, including growth of donor plants, anther staging and bud selection, bud surface sterilization and anther extraction, culture media preparation, anther incubation, and culture of embryos and plant regeneration, were conducted as described by Keller (1984) with exceptions noted. Two alterations of the anther culture

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media from the standard protocols included the addition of 14% instead of 10% sucrose, and the addition of 2 mg·L^{-1} $AgNO_3$. F_1 hybrid plants that served as anther donors were grown in a GC-15 plant growth chamber (Environmental Growth Chambers, Chagrin Falls, Ohio) with a 16-h photoperiod and temperature of 15 °C day/10 °C night. In Spring 1996, broccoli anther cultures were initiated using the F_1 hybrids 'Everest', 'Sultan', 'Arcadia', and 'Viking' as anther donors. The entire process from initiation of anther cultures to establishment of microspore-derived, regenerated plantlets took 4 to 5 months. During late Summer 1996, conditioned broccoli regenerants were potted and moved to a temperature-controlled greenhouse without supplemental light, where they were grown to maturity during the fall. Regenerants were grown in 25.4-cm-diameter pots filled with a commercial potting mix (Metromix 360; Grace Sierra, Milpitas, Calif.). Each broccoli regenerant grown in the greenhouse originated from an individual embryo in culture, and no two regenerants descended from the same embryo.

Plant materials for ploidy determination. About 400 plants (with variable genetic background) were regenerated from the cycle of anther culture described above. A section of leaf was taken for cytometric analysis from each plant at the four- to five-leaf (seedling) stage by cutting a disk (18-mm diameter) from the third leaf from the apex using a cork borer. The sampled leaf was still expanding rapidly. Of the ≈400 regenerants evaluated, 140 individuals were chosen for analysis a second time at a mature plant stage. The 140 plants that were sampled at both the seedling and mature stages represented a mix of haploids (33), diploids (57), tetraploids (47), and putative triploids (3) identified in the seedling-stage analysis. This group of plants included diploids and tetraploids chosen at random; however, all haploids and triploids identified initially were resampled because they represented a smaller percentage of all regenerants (≈8% and 1%, respectively), and we wanted to maximize their numbers in this study. In the second analysis of the 140 identified regenerants, a section of leaf (fully expanded) from the top third of each plant was taken at the time of heading as described above.

Flow cytometry. Leaf samples from broccoli regenerants were prepared following the DNA flow cytometry protocol described by Arumuganathan and Earle (1991b). Preparation of samples involved: 1) slicing leaf sections with a razor blade in $MgSO_4$ buffer containing propidium iodide to release and stain intact nuclei; 2) filtering of homogenates; 3) centrifuging filtrates; and 4) resuspending and continued staining of pelleted nuclei in the same buffer. The relative DNA content of nuclei suspensions and, indirectly, the ploidy of sampled plants, were determined by measuring fluorescence of preparations of propidium iodide-stained nuclei using an EPICS XL flow cytometer (Coulter Electronics, Hialeah, Fla.) with an argon-ion laser operating at a wavelength of 488 nm. Nuclei were analyzed for fluorescence intensity, and a his-

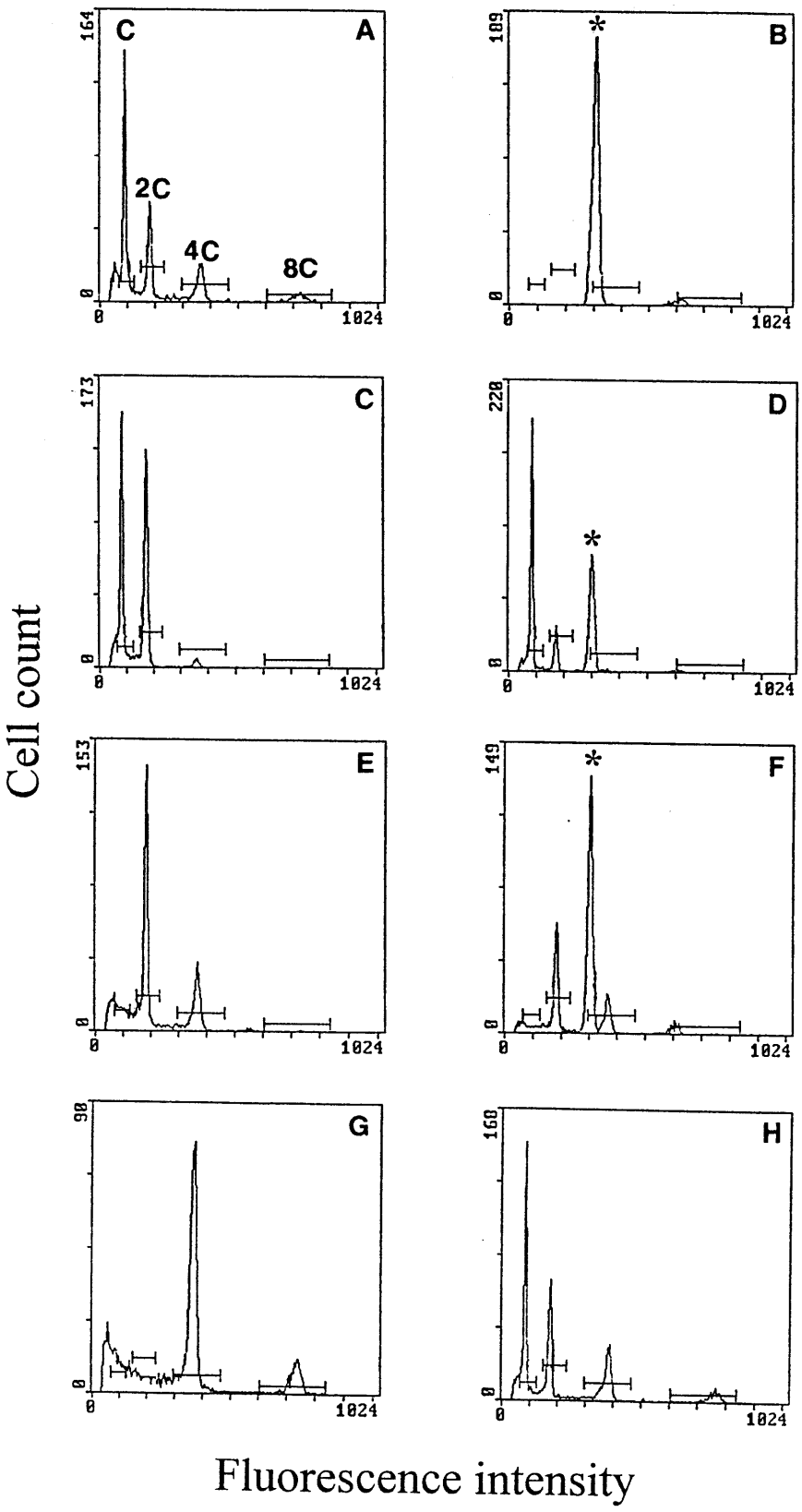

Fig. 1. DNA histograms from the flow cytometer of individual samples or regenerants. (**A**) Control sample derived from equal amounts of leaf material from a known haploid, diploid and tetraploid; (**B**) a control sample of chicken red blood cells (CRBC) alone; (**C**) a representative haploid alone or (**D**) with CRBC as an internal standard; (**E**) a representative diploid alone or (**F**) with CRBC as an internal standard; (**G**) a representative tetraploid alone or (**H**) with a haploid control as an internal standard. C, 2C, 4C, and 8C peaks are marked in **A**. Horizontal bars on each graph indicate ranges in positions of the C, 2C, 4C, and 8C peaks. The position of the G_0+G_1 phase cell peak of CRBC is indicated by "*".

togram was generated in which frequency (number of nuclei) of a particular intensity was plotted vs. intensity. With each preparation from an individual regenerant, the cytometer was set to count 5000 nuclei. Flow cytometry was performed the same day as sample preparation.

Forty to 50 samples were assayed in a single cytometry session. Each session included controls consisting of leaf samples from known broccoli haploids, diploids, and tetraploids, and a standard preparation of chicken red blood cells (Pocono Rabbit Farm and Laboratory, Canadensis, Pa.) run alone and in combination with a diploid control. At the start of each session, the flow cytometer was adjusted, if necessary, and haploid, diploid, and tetraploid peak positions were set at approximate fluorescence intensities of 90, 180, and 360 units, respectively. After making initial flow cytometer adjustments, none were made during the rest of the session. From session to session, flow cytometer settings (e.g., photomultiplier tube voltage adjustment) were usually the same. Haploid and tetraploid controls had been found initially in preliminary runs in 1995. In those runs, standard diploid broccoli cultivars were compared with regenerants of unknown ploidy. Individuals exhibiting half or twice the fluorescence of diploids were identified, and the ploidy of these putative haploids and tetraploids, respectively, was confirmed using cytological evaluation of pollen mother cells (Keller, 1984). The confirmed haploids, diploids, and tetraploids have been maintained vegetatively in our program since their identification in 1995 and continue to serve as controls in our flow cytometry analysis.

DNA histograms from the flow cytometer for the unknown samples were compared with those for controls, and characteristic haploid, diploid, tetraploid, and other fluorescence intensities were identified (Fig. 1). Unknowns were classified based on these comparisons. In previous work, ≈95% of individual regenerants from anther culture were characterized as either haploid, diploid, or tetraploid, with haploids the least frequent of these three types (Farnham, 1996). Histograms of analyzed individuals exhibit two peaks of fluorescence intensity, one primary peak that corresponds to G_0+G_1 phase cells and a secondary peak that corresponds to G_2+M phase cells. The relative peak size reflects the number of cells in the respective phases. Analysis of individual histograms permits determination of the number of cells in each phase. For all 140 regenerants sampled at the seedling and heading stages, the ratio of the number of cells in the G_0+G_1 phase to the number in the G_2+M phase (the cell phase ratio) was computed. Ploidy and plant stage effects on cell phase ratio were determined by analysis of variance (ANOVA) using PROC GLM of SAS (SAS). Individual plants were considered nested within a given ploidy level, and stage nested within individual plants.

Stability of flow cytometry preparations. A total of 12 leaf samples taken at the heading stage, plus controls, were prepared for flow cytometry analysis as described previously. Plants sampled included a mix of haploids, diploids, tetraploids, and triploids. Preparations were stored at 4 °C in a refrigerator for 7 d. A subsample of each preparation was run through the flow cytometer on the day of preparation and also after 1, 2, 4, and 7 d in cold storage. Ploidy determination and characteristics of histograms were evaluated for each day. This experiment was repeated with a different sample of regenerants.

Stability of whole leaves. A single, recently fully expanded leaf was removed from each of 12 regenerants (a mixture of ploidy types) and the controls at heading stage, by cutting the petiole with a razor blade just above attachment to the main stem. The petiole of each leaf was wrapped in a moistened paper towel and placed in a plastic bag with a zip lock. All bags with leaves were stored at 4 °C in a refrigerator for 7 d. Tissue was cut from each leaf on the day it was removed from the plant, and also 1, 2, 4, and 7 d after the leaf was placed in cold storage. On each day the stored leaves were analyzed, sections were prepared, and flow cytometry was conducted as indicated previously. Ploidy determination and characteristics of histograms were evaluated for each day of flow cytometry. This experiment was also repeated with a different sample of regenerants.

Determination of DNA content. Quantification of the absolute amount of nuclear DNA in haploids, diploids, tetraploids, and triploids was determined using the methods of Arumaganathan and Earle (1991b). Samples representing 35 individual confirmed haploids, 35 diploids, 35 tetraploids, and three triploids were analyzed to obtain DNA content for each ploidy type. Chicken red blood cells (CRBC) were used as an internal standard for comparing peak positions of haploids and diploids. However, the CRBC and triploid and tetraploid peaks overlapped, so a haploid broccoli control was used as an internal standard to quantify these types. To compute DNA content per nucleus for broccoli samples, the following formula was used when CRBC served as an internal standard:

$$\text{pg DNA per nucleas} = \frac{\text{mean position of broccoli peak}}{\text{mean position of CRBC peak}} \times 2.33 \text{ pg DNA per CRBC nucleas.}$$

With an internal haploid standard, the formula was changed so that mean position of the CRBC peak was replaced with mean position of the haploid peak, and 2.33 pg DNA was replaced with 0.67 pg DNA per haploid nucleus.

Table 1. Mean squares (MS) from analysis of variance for cell phase ratio (no. of G_0 plus G_1 phase cells/no. of G_2 plus M phase cells) of anther culture-derived regenerants with variable ploidy determined by flow cytometry.

Source	df	MS
Ploidy	3	358.3**
Plant (ploidy)	136	14.2
Stage	1	12.5
Ploidy × stage	3	52.7**
Error	136	11.6
Corrected total	279	

**Significant at $P < 0.01$.

Results and Discussion

Mean DNA contents (±SE) determined by flow cytometry were 0.67 ± 0.01 pg per haploid (C) nucleus, 1.35 ± 0.01 pg per diploid (2C) nucleus, 2.04 ± 0.03 pg per triploid (3C) nucleus, and 2.67 ± 0.01 pg per tetraploid (4C) nucleus. The direct estimates of DNA content for haploids, triploids, and tetraploids were very close to expected, relative to content of diploids. We are unaware of other reported direct measurements of DNA content of euploids, other than diploids, in *B. oleracea*. Our estimate of DNA content of diploid broccoli is comparable to that reported for *B. oleracea* by Arumaganathan and Earle (1991a). Those authors obtained estimates of 1.30 to 1.37, 1.25, 1.30, and 1.26 pg DNA per 2C nucleus, for one to four varieties each of cauliflower, cabbage, brussels sprouts and broccoli, respectively.

Out of 140 regenerants analyzed at seedling and mature plant stages, 139 were classified as having the same relative peak positions on histograms, and thus the same ploidy levels, at both stages. One individual exception was classified as an aneuploid at the seedling stage and a tetraploid at the mature stage. Results of ploidy determination using flow cytometry as outlined in this research indicate an accuracy rate >99% when the two separate samples (taken at different stages) are considered duplicates of the same plant. This represents a very reliable method of ploidy determination.

Athough determination of ploidy was essentially the same between sampled stages, ANOVA indicated some statistically significant effects of plant variables on cell phase ratios resulting from flow cytometry (Table 1). Combined statistical analysis of all individuals indicated a significant effect of ploidy on cell phase ratio, but no plant stage effect. However, a significant stage × ploidy interaction was indicated. Separate analyses of individuals within a given ploidy level showed that the stage × ploidy interaction is explained by a significant stage effect within diploids and tetraploids. Cell phase ratios were lowest for haploids at 2.1 and 1.9 for the seedling and mature stages, respectively, and greatest for tetraploids at 8.1 and 5.8 for the same respec-

Table 2. Cell phase ratio (no. of G_0 plus G_1 phase cells/no. of G_2 plus M phase cells) means of haploids, diploids, triploids and tetraploids analyzed by flow cytometry at seedling and mature plant stages.

		Plant stage	
		Seedling	Mature plant
Ploidy	No. plants	Cell phase ratio	
Haploid	33	2.1	1.9
Diploid	57	3.0	4.1*
Triploid	3	6.4	5.0
Tetraploid	47	8.1	5.8*

*Significantly different from and seedling stage at $P < 0.05$.

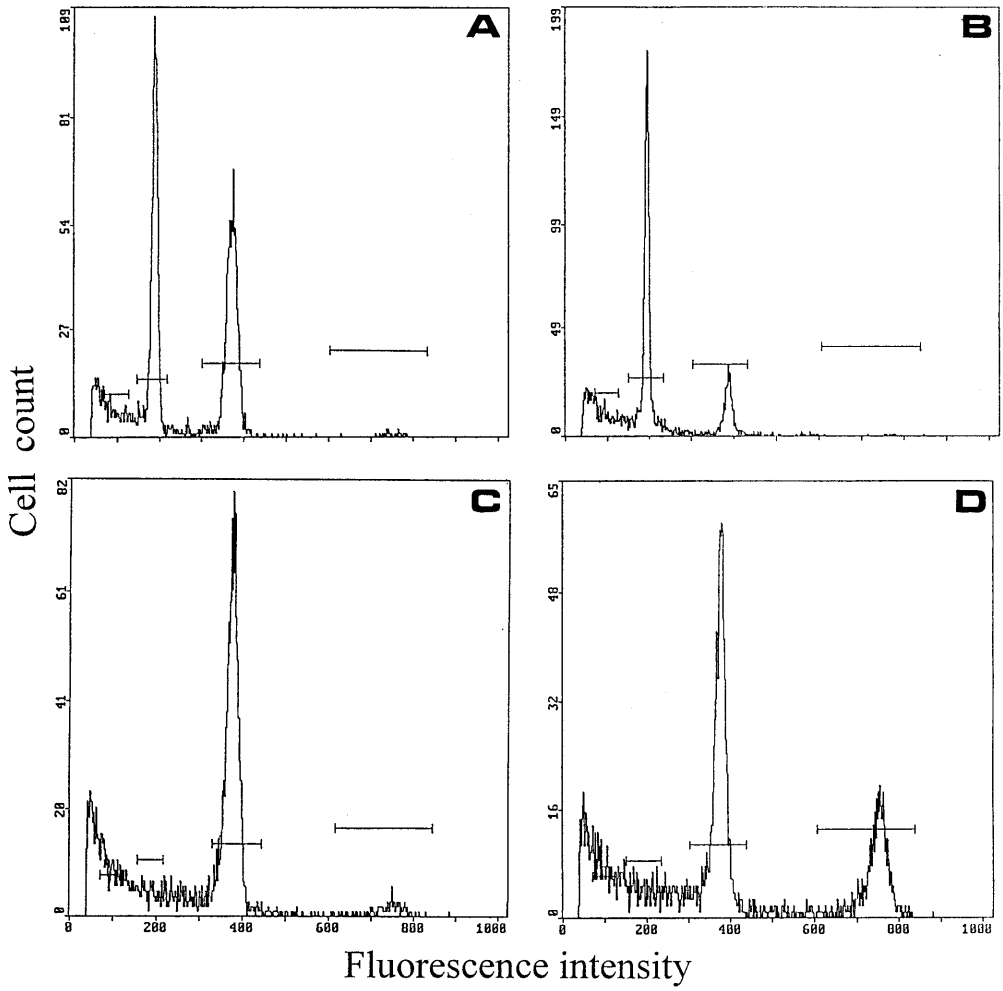

Fig. 2. DNA histograms from the flow cytometer of two individual regenerants sampled at two different stages: a representative diploid sampled at the (**A**) seedling stage and at the (**B**) heading stage; a representative tetraploid sampled at the (**C**) seedling and (**D**) heading stages. Horizontal bars on each graph indicate ranges in positions of the C, 2C, 4C, and 8C peaks from left to right, respectively.

tive stages (Table 2). With diploids, cell phase ratios were usually higher (i.e., fewer cells in G_2+M phase) at the mature plant stage than at the seedling stage (Fig. 2 a and b). Conversely, the cell phase ratios with tetraploids were usually higher at the seedling stage than at the mature plant stage (Fig. 2 c and d). These results indicate that DNA histograms may exhibit a different appearance at seedling vs. mature plant stages due to relative differences in the number of cells in G_0+G_1 phase vs. the number of cells in G_2+M phase. However, peak positions (fluorescence intensity) for individuals, relative to controls, are more important identifying characteristics for ploidy determination, and, thus, the observed differences in cell phase ratios will not impact ploidy typing of regenerants.

All flow cytometry preparations stored at 4 °C remained stable for 2 d after they were made. After storage for 7 d, all controls and a majority of samples were still stable in two separate trials, and resulting ploidy determinations were unchanged. However, in the first trial, peak positions of two samples (one haploid and one tetraploid) began to shift significantly to a lower fluorescence intensity between 2 and 4 d after preparation, and in the second trial, one sample (tetraploid) began shifting to a lower intensity during the same time period (Fig. 3). In all three cases where peaks shifted from control positions, individuals would have been misclassified as aneuploids as a consequence of storing preparations too long. There was no effect of storing preparations for up to a week on cell phase ratios of DNA histograms.

Storing whole leaves from regenerants at 4 °C had no effect on ploidy determination or cell phase ratios of DNA histograms up to 7 d after leaves were excised from plants. In two separate trials, all control and individual leaf preparations from regenerants were unchanged when sampled 1, 2, 4, and 7 d from initial analysis and placement at 4 °C. Thus, these results clearly show that if one excises leaves, wraps petioles in moistened paper towels, and stores the leaves in sealed plastic bags, delayed preparation of leaves and ploidy determination by flow cytometry is effective and accurate.

Once plants are removed from the culture process and seedlings are established, our results indicate that ploidy level is established and plants probably will not change as they grow and mature. Polyploidization that occurs during anther culture must occur sometime after anthers are first cultured, but before seedlings are established. There are several steps, including microspore embryogenesis, embryo culture, and shoot regeneration, during which polyploidization might occur. A primary advantage of flow cytometry for ploidy determination is that it can be conducted on seedlings recently removed from culture. By determining ploidy at this early stage, a breeder can identify diploids and eliminate nondiploids. This saves both time and the space required to grow plants to maturity. Although characteristics of guard cells in the leaf epidermis are correlated with ploidy and can be used effectively to characterize regenerants (Ockendon, 1986), this method requires mature leaves, not actively expanding leaves like those of seedlings. We have also correlated guard cell characteristics (e.g., guard cell length) of mature leaves with ploidy of regenerants (unpublished data), but like Ockendon (1986) we have observed significant overlap in characteristics between haploids and diploids and between diploids and tetraploids. This overlap increases the error in typing ploidy of regenerants. Overall, we have found that typing of plants using guard cell characteristics

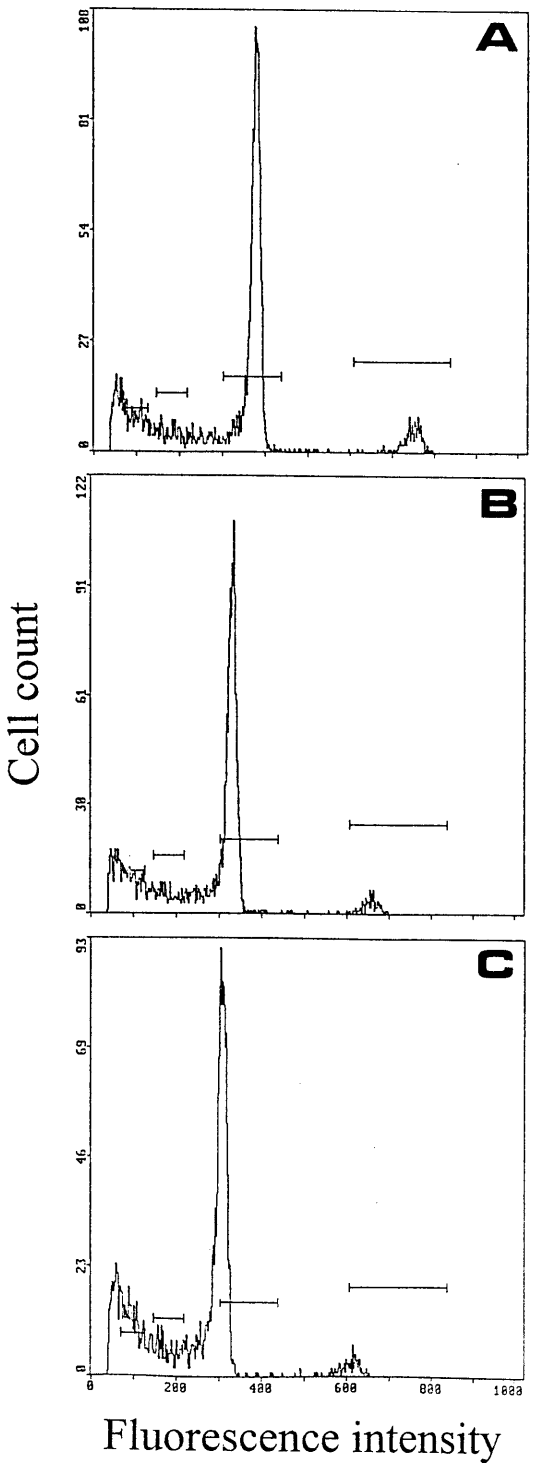

Fig. 3. Effect of storage at 4 °C on DNA profile of an individual tetraploid preparation. The sample was analyzed on the flow cytometer on the (**A**) day of preparation, and also after (**B**) 4 and (**C**) 7 d in cold storage. Horizontal bars on each graph indicate ranges in positions of the C, 2C, 4C, and 8C peaks from left to right, respectively.

is more costly than flow cytometry (<$2 U.S. per sample) due to the amount of time required for microscopic observations.

The limiting factor that prevents most broccoli breeders from using flow cytometry to determine ploidy of anther-derived plants is the cost of obtaining and maintaining a flow cytometer. In addition, most breeders have no experience with flow cytometry technology. Results of this study indicate that breeders could determine ploidy of regenerated populations by processing leaves, maintaining preparations on ice, and sending them by overnight delivery to a contract laboratory offsite, as long as analysis was done within 1 or 2 d after preparation. Alternatively, whole leaves could also be kept cold, sent offsite, and prepared and analyzed at another location. In this latter case, even if the entire process takes a week to complete, results of ploidy determination would still be reliable.

The accuracy of flow cytometry, as illustrated in this paper, along with other advantages we have outlined, make this method a very effective and reliable way to determine ploidy of anther-derived broccoli. Use of this technology should improve the overall efficiency of identifying DH individuals in populations of plants regenerated from anther culture.

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