Instability of Candida albicans Hybrids

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Total cellular DNA content, determined by a colorimetric method, was used as an index of ploidy in Candida albicans. Mononucleate hybrids were formed by fusion of spheroplasts derived from diploid parent strains. Five hybrids, of six studied, were taken to be tetraploid on the basis of estimated DNA content. One hybrid was taken to be hexaploid or near-hexaploid. Selection for increased resistance to 5-fluorocytosine in the hybrids, which were heterozygous for resistance, resulted in isolation of variants which were of lower ploidy than the hybrids from which they originated. Variants were obtained which corresponded (in measured DNA content) to aneuploid, triploid, and diploid states. These results may form the basis of a cyclic parasexual system (2n × 2n→4n→2n) for genetic analysis of this asexual species.

Recent studies indicate that typical Candida albicans isolates are diploid or near-diploid (9, 12, 15–19). Induced mitotic recombination has provided a useful initial method of genetic analysis in this amitotic species; mitotic recombination and gene linkage have been demonstrated by methods which do not involve hybridization. However, mitotic recombination analysis is of limited value in the absence of methods which allow construction and analysis of hybrids.

Various workers (2, 4–6, 8, 10, 11, 13, 14) have employed spheroplast fusion to generate C. albicans hybrids, and this method is now well established. It seemed useful to attempt to construct a cyclic parasexual system based on hybridization and subsequent reduction in ploidy (2n × 2n→4n→2n) as a general method for genetic mapping and construction of useful recombinant strains. We supposed that a hybrid strain which was heterozygous for some recessive resistance marker might become resistant by loss of a chromosome bearing the allele for sensitivity. The resultant aneuploid strain might then undergo further chromosome loss and arrive at a physiologically balanced state (in terms of the ratio of crucial genes) detectable as a net decrease in ploidy. Then, selection for resistance appeared to offer the possibility of ready selection for reduction in ploidy, a necessary step in construction of a cyclic parasexual system.

We report here the construction and properties of hybrids which were heterozygous for resistance to the clinical antifungal agent 5-fluorocytosine (5-FC). The hybrids were unstable and gave rise to resistant variants. Some resistant variants were of a lower ploidy than the hybrids from which they arose, as assessed by determination of DNA content per cell; variants were obtained with DNA contents similar to those of the (diploid or near-diploid) parent strains which fused to form hybrids. These results provided the essential elements of a cyclic parasexual system.

MATERIALS AND METHODS

Strains. Clinical isolates AD5, AD11, AD13, and AD18 were obtained from New Addenbrookes Hospital, Cambridge. Clinical isolates QC6 and QC12 were obtained from Queen Charlotte’s Maternity Hospital, London. Parent strains for fusion (Table 1) were derived from clinical isolates by UV-induced segregation by established methods (17, 18).

Parent strain 85A was a histidine auxotroph obtained from isolate AD5. The other parent strains were methionine auxotrophs (MetA phenotype [18]) obtained from the other clinical isolates. Parent strain 85A was (homozygous) sensitive to 5-FC; the other parents were heterozygous for resistance.

Homozygosity and heterozygosity (for resistance to 5-FC) were inferred from the resistance phenotypes determined under standard conditions (see below) which were similar to those described previously (1, 16). No test was made for allelism among the met markers borne by parent strains.

Hybridization. The method of Poulter et al. (10) was used to obtain spheroplasts and induce fusion. Prototrophs (possible hybrids) were selected in MIN agar (3).

5-FC resistance phenotypes. Cells (ca. 10⁶ CFU) were spread on HMLF agar; eight strains were tested on each plate (diameter, 90 mm). HMLF agar was MIN agar supplemented with l-histidine, l-methionine, l-lysine, and 5-FC. Each of the amino acids was present at a final concentration of 40 μg/ml; 5-FC was present at a final concentration of 50 μg/ml. Filter-sterilized 5-FC (Sigma Chemical Co.) was added to autoclaved medium. Cultures were incubated at 37°C, and the phenotypes (sensitive, partially resistant, highly resistant) were assessed as described previously (16).

Isolation of resistant variants. For isolation of resistant variants from a hybrid, a clone was suspended in water and a suitable sample was spread on YEPD agar (3) to yield ca. 150 colonies. After incubation at 37°C for two days, the culture was replica-plated on HMLF agar (primary replica). The primary HMLF replicas of colonies grew slowly and gave rise to papillations (presumptive resistant variants). The primary replica was replica plated on HMLF agar (secondary replica), and small colonies corresponding to the papillations (above) were observed after incubation for 2 days. The majority of colonies present on the YEPD master plate gave rise to one (or a few) papillation on the primary replica and resultant small colonies on the secondary replica. Presumptive resistant variants present on the secondary replica were purified for determination of DNA content. Determination of the 5-FC resistant phenotype indicated that...
TABLE 1. Hybrids H1 through H6; genotypes of parent strains

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Parents</th>
<th>Genotypes</th>
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<tbody>
<tr>
<td>H1</td>
<td>85A × 1083</td>
<td>MET his FCY × met HIS FCY</td>
</tr>
<tr>
<td>H2 through H5</td>
<td>_b</td>
<td>MET his FCY × met HIS fcy</td>
</tr>
<tr>
<td>H6</td>
<td>85A × 1137C</td>
<td>MET his FCY × met HIS fcy</td>
</tr>
</tbody>
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a Shown are the genotypes of the parent strains at the loci MET, HIS, and FCY. Dominant alleles are shown in upper case and recessive alleles are shown in lower case. The HIS locus was linked to the FCY locus (Whelan and Markie; submitted for publication; see the text). MET-FCY linkage was not detected (see the text).

b Hybrids H2 through H5 were constructed by fusing parent strain 85A with parent strains which were phenotypically similar to parent strain 1083; genetic similarity (allelism) to strain 1083 was not tested. Parent strain 85A was derived from clinical isolate AD5, and the other parents of hybrids H1-H5 were derived from other clinical isolates (AD1, AD18, AD13, QC6, QC12). Parent strain 1137 was a spontaneous homozygous resistant segregant derived from the heterozygous (FCY/fcy) parent of hybrid H5.

days at 33°C in liquid YEPD medium (5 ml) shaken on a Vibraflex mixer. Cultures were diluted (1:10) in dust-free saline (0.9% NaCl [wt/vol]), mixed for 1 min on a vortex mixer, and then immersed in a Dawes Sonibath for 15 s. Microscopic examination of suspensions indicated that more than 95% of cells were singlets. The suspensions were diluted (1:100) with saline and analyzed for cell size with a Coulter Counter (model ZM) fitted with a 30-μm orifice tube and calibrated with monodisperse latex beads. The volumes of ca. 2 × 10⁶ cells were determined for each sample. The Coulter Counter was linked to a computer (Acorn BBC microcomputer) to provide automatic data reduction and cell-size distribution plots. Correction for the difference in conductivity between C. albicans cells and the standard latex beads was accomplished by photomicrographic comparison of cells (parent strain 1083 and hybrid H1) with standard latex beads.

RESULTS

Hybridization. Hybrids with the desired genotype at the locus (FCY) which determined resistance to 5-FC were obtained by fusion of auxotrophic spheroplasts by the method of Poulter et al. (10) followed by selection for prototrophs. The auxotrophic parent strains were derived from clinical isolates and were fused in the combinations shown in Table 1.

The prototrophs obtained after fusion and selection were screened to distinguish hybrids from prototrophic revertants of the parent strains. The initial screening was based on the expectation that hybrids derived from the present fusions would differ from both parents in resistance to 5-FC. Hybrids H1 through H5 resulted from FCY/FCY × FCY/fcy fusion aimed at generating the FCY/FCY/FCY/fcy genotype. Previous studies (1, 16) show that FCY/fcy heterozygotes are more resistant than sensitive (FCY/FCY) homozygotes but less resistant than resistant (fcy/fcy) homozygotes, and another study shows that partial resistance (FCY/fcy) strains is associated with decreased UMP pyrophosphorylase activity (about one-half the specific activity found in FCY/FCY strains), whereas high resistance (fcy/fcy) strains is associated with a barely detectable activity (16a). Those studies suggest that resistance in tetraploid hybrids would be determined by the ratio of fcy to FCY alleles in the genome. On this basis, putative hybrids H1 through H5 were chosen as being more resistant than the FCY/FCY parent and less resistant than the FCY/fcy parent.

Putative hybrid H6 (FCY/FCY × fcy/fcy fusion) was readily isolated by the foregoing rationale; it displayed the resistance phenotype of typical FCY/fcy heterozygotes and differed markedly from both parents.

In addition to prototrophs such as H1 through H6, which were chosen as putative hybrids solely on the criterion of displaying the expected 5-FC resistance phenotype, prototrophs which appeared to result from reversion were also found. A prototroph which resembled one of the parent strains in 5-FC resistance phenotype was taken to indicate reversion of the auxotrophic marker of the parent; these occurred at a frequency similar to that of putative hybrids.

DNA content of parents and hybrids. The mean DNA content of the parent strains was used as the base datum (expressed in femtograms per cell and denoted P in Fig. 1) against which changes in ploidy were estimated. For the seven parent strains (Table 1), the mean DNA content (± standard deviation) was 37.9 ± 0.8 (range, 36.7 to 38.8), and this value was in reasonable agreement with the best current estimates of the DNA content of typical C. albicans.

all presumptive resistant variants subjected to DNA determination were more resistant than the hybrids from which they originated. The majority (65%) of the variants studied were stable, and the remainder gave rise to variants which were more resistant.

To obtain resistant variants from parent strains, independent colonies were suspended in water, and cells (ca. 10⁶ CFU) were spread on HMLF agar (eight clones per plate). Resistant colonies which appeared against a slow-growing background lawn of cells after incubation for 3 to 4 days were purified on YEPD agar and shown to be resistant. One resistant variant was taken from each independent colony of the parent strain for DNA determination.

DNA determination. Cultures were grown in liquid YEPD medium for 3 days at 37°C with shaking. A sample of culture was diluted, and the number of cells per milliliter of culture was determined by a hemacytometer count of at least 400 cells. Buds were counted as cells. Another sample (10 ml) was subjected to DNA determination by method B of Riggsby et al. (12), using the diphenylamine color reaction with highly polymerized calf thymus DNA (British Drug Houses) as standard. For any one culture of a strain, the DNA content was obtained from the mean of two determinations of DNA (on duplicate samples of the centrifuged perchloric acid extracts [12]) divided by the mean of two or more estimates of cell number. In some cases (see below), independent estimates of DNA content were made by repeating the above procedure on another culture grown from the same clone.

Reproducibility of estimation of DNA content was assessed by calculating deviations from the mean of two or more independent estimates for a strain; the deviations were expressed as a percent fraction of the mean value for the strain. For 11 strains, which included representative parents, hybrids, and resistant variants, deviations ranged from 0.3 to 13.3% of the mean values for the strains, and the average deviation was 6.7% of the mean.

Nuclear stain. Cells from overnight YEPD agar cultures were suspended in 70% ethanol (vol/vol in water) and left at room temperature for 30 min. The suspension (1 ml) was centrifuged, and the cells were washed with water (1 ml). The pellet was suspended in DAPI solution (2,4-diamidino-2-phenylindole [Sigma]; 0.5 g/ml in water) and cells were examined with a fluorescence microscope equipped with filters of the type suggested by Williamson and Fennell (20).

Determination of cell volume. Cultures were grown for 3 days at 33°C in liquid YEPD medium (5 ml) shaken on a Vibraflex mixer. Cultures were diluted (1:10) in dust-free saline (0.9% NaCl [wt/vol]), mixed for 1 min on a vortex mixer, and then immersed in a Dawes Sonibath for 15 s. Microscopic examination of suspensions indicated that more than 95% of cells were singlets. The suspensions were diluted (1:100) with saline and analyzed for cell size with a Coulter Counter (model ZM) fitted with a 30-μm orifice tube and calibrated with monodisperse latex beads. The volumes of ca. 2 × 10⁶ cells were determined for each sample. The Coulter Counter was linked to a computer (Acorn BBC microcomputer) to provide automatic data reduction and cell-size distribution plots. Correction for the difference in conductivity between C. albicans cells and the standard latex beads was accomplished by photomicrographic comparison of cells (parent strain 1083 and hybrid H1) with standard latex beads.

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isolates obtained by Riggsby et al. (12) by the method employed here.

The estimated DNA content of each of five putative hybrids was in satisfactory agreement (within 12%) with the value predicted by summing the parental values (Fig. 1); these results were taken to confirm hybridization. The DNA content of one putative hybrid (H5; Fig. 1) was significantly higher in three independent estimates (88.8, 109, and 114 fg per cell) than the value predicted by summing the parental values (36.7 + 37.8). This exceptional strain is believed to be a hybrid which may have resulted from fusion of three nuclei, but it is also possible that unknown events resulted in an increased DNA content after fusion of two nuclei.

Microscope examination of cultures stained with the fluorescent nuclear stain 4,6-diamidino-2-phenylindole (20) revealed that the hybrids were mononucleate.

Instability of hybrids. Hybrids H1 through H6 gave rise to variants which displayed increased resistance to 5-FC (see above). The sample of resistant variants was heterogeneous in terms of resistance; some variants were highly resistant and stable, whereas others were less resistant and gave rise to further variants which were more resistant. In addition, some resistant variants expressed a parental marker: 17 methionine auxotrophs and 3 histidine auxotrophs were found among 188 resistant variants isolated from the hybrids. In contrast, no auxotrophs were found when 2,313 colonies were grown from cells of hybrids H1 through H6 on nonselective medium (YEPD agar) and replica plated on minimal medium. These results suggest that variants which displayed increased resistance to 5-FC arose in a process which significantly increased the probability that the met marker would become homozygous (or hemizygous).

DNA content of resistant variants. We supposed that hybrids H1 through H6 might give rise to resistant variants by three distinct processes: de novo mutation, mitotic recombination, or loss of one or more FCY alleles due to loss of the relevant chromosome(s). The contribution of each of these processes could not be assessed by analysis of the variants by using available genetic methods. However, variants in which multiple chromosome loss resulted in a detectable decrease in ploidy might be detected by determination of cellular DNA content.

Among 27 resistant variants examined (Fig. 1), 8 did not differ markedly from the hybrids in cellular DNA content; the mechanism by which these variants arose is not known. Thirteen variants were found to be intermediate in DNA content, corresponding to triploid and aneuploid states between diploid and tetraploid. These include 1 variant of the exceptional hybrid H5 and 12 variants (with DNA values in the range 42 to 64 fg per cell) isolated from the other hybrids. Six resistant variants were found to be indistinguishable in DNA content from the parents fused to form the relevant hybrids; at least one variant of this kind (diploid or near-diploid) was obtained from each hybrid except H3.

The foregoing results indicate that acquisition of increased resistance to 5-FC was associated with a significant decrease in cellular DNA content, which we take to mean a detectable decrease in ploidy, in about 70% of the variants examined.

DNA content of resistant variants isolated from FYC/FCY heterozygotes. It seemed possible that typical FYC/FCY heterozygotes, such as the diploid (or near-diploid) clinical isolates from which the present parent strains were derived, might undergo a marked reduction in ploidy associated with acquisition of resistance to 5-FC. Although previous studies (1, 16, 16a) have shown that FYC/FCY heterozygotes give rise to resistant variants by mitotic segregation (FYC/FCY + FCY/FCY), those studies do not exclude the possibility that resistant variants may also result from loss of the relevant chromosome and generalized reduction in ploidy.

To test for a generalized reduction in ploidy, independent resistant variants were isolated from strains 1083 and 145 (the met/met FYC/FCY parents of hybrids H1 and H3, respectively), and cellular DNA content was estimated. The results (mean ± standard deviation, 37.5 ± 2.0; range, 34.2 to 42.7 fg per cell) did not rule out the possibility that some variants resulted from loss of one or a few chromosomes but did indicate that no marked reduction in ploidy was associated with acquisition of resistance in this sample (nine variants from strain 1083 taken with eight variants from strain 145). Detectable decrease in ploidy was observed significantly more frequently in the sample of variants obtained from hybrids (see above) than in the sample obtained from diploid (or near-diploid) FYC/FCY heterozygotes (χ² > 6.7; P < 0.01).

Relationship of cell volume and DNA content. For these experiments cultures were grown aerobically in YEPD medium to stationary phase. Dilution in saline, followed by vigorous mixing and mild sonication, yielded suspensions which were composed predominantly of single unbudded cells (see above). Typical cell volume distributions determined with a Coulter Counter were bimodal (Fig. 2). The peak at lower volume was taken to represent the distribution of volumes of single unbudded cells, and the mean value of

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**FIG. 1.** DNA content of parents, hybrids, resistant variants. Shown for each hybrid is the DNA content found for the parents (A), the hybrid (B), resistant variants which were prototrophic (C), and resistant variants which required methionine for growth (D). Each point represents the DNA content of a different strain determined as described in the text. Strain 85A was a parent in all crosses; its DNA content (36.7 fg per cell) is shown only once in the figure (with hybrid H1). The symbol P denotes the mean DNA content of the parents (37.9 fg per cell).
this peak, corrected for the difference in conductivity of \textit{C. albicans} cells relative to the latex bead calibration standard (see above), was taken to be the fundamental volume for the strain. The peak at higher volume presumably resulted from the presence of doublets and multilets in the suspensions, in addition to a contribution from passage of two or more singlets through the orifice within the resolution time of the instrument.

The fundamental cell volumes of selected parent strains, hybrids, and resistant variants were determined. The relationship of cell volume to DNA content was linear (Fig. 3) over a range of values for DNA content from diploid to approximately hexaploid.

**Linkage.** The present study permitted some preliminary linkage assignments. It was assumed, based on findings in other parasexual systems (7), that markers borne on the same chromosome segregated as a group during reduction in ploidy. As an initial test of whether the \textit{MET} and \textit{FCY} genes were borne on the same chromosome, only variants which were demonstrably of lower ploidy than the relevant hybrid and which were highly resistant and stable for resistance were considered. These variants presumably corresponded to homozygous (\textit{fcy/fcy/fcy/fcy/fcy}) or hemizygous (\textit{fcy}) states at the resistance gene. Consideration of genotypes of parents (Table 1) indicated that all such variants would express the \textit{met} marker if the \textit{MET} gene and the \textit{FCY} gene were borne on the same chromosome, whereas most would be prototrophic if these genes were borne on heterologous chromosomes. Two prototrophic variants which satisfied the foregoing requirements of ploidy and resistance were obtained from hybrid H1, and two similar variants were obtained from hybrid H3 (ploidy data in Fig. 1; resistance data not shown). These results indicated that the \textit{MET} gene and the \textit{FCY} gene were borne on heterologous chromosomes in the parents of these hybrids.

A further test for \textit{MET-FCY} linkage was provided by consideration of all methionine-requiring resistant variants which displayed a demonstrable decrease in ploidy. Expression of the Met phenotype was taken to indicate hemizygosity (\textit{metl/metl/metl/metl}) or hemizygosity (\textit{metl}). Consideration of the genotypes of hybrids H1 through H5 (Table 1) indicated that all Met auxotrophs (except those which were hemizygous) were expected to be heterozygous at \textit{FCY} and, therefore, unstable for resistance if \textit{MET} and \textit{FCY} were borne on the same chromosome. However, methionine-requiring resistant variants which resulted from reduction in ploidy (Fig. 1) and which were highly resistant and stable for resistance (data not shown) were found; one such variant was obtained from each of three hybrids (H2, H3, H5), and two were obtained from another hybrid (H4). These results suggest that the \textit{MET} gene and the \textit{FCY} gene were borne on heterologous chromosomes in all parent strains (Table 1); further studies, with a larger sample of prototrophic resistant variants and including genetic characterization of variants to test for hemizygosity, are required to test this hypothesis.

The foregoing results provided preliminary verification of the initial assumption of this study: that \textit{MET} and \textit{FCY} were borne on heterologous chromosomes and that expression of the \textit{met} marker would therefore be a useful criterion by which variants which had undergone a generalized reduction in ploidy might be chosen from the sample of resistant variants. The finding (Fig. 1) that 14 methionine-requiring resistant variants (of 15 tested) were significantly reduced in ploidy validated the use of an unlinked marker in finding useful variants.

Histidine-requiring resistant variants were obtained significantly less frequently in the present study than were methionine-requiring resistant variants (see above). Essentially identical frequencies of these two auxotrophs were expected if \textit{HIS}, \textit{MET}, and \textit{FCY} were borne on heterologous chromosomes. The apparent systematic partial exclusion of His auxotrophs from the sample of resistant variants suggested the genotype shown in Table 1 for strain 85A. The indicated \textit{HIS-FCY} linkage was consistent with the foregoing result because selection for resistance would select for the \textit{HIS} allele rather than the \textit{his} allele. In studies to be reported elsewhere, it will be shown that the \textit{his} marker of strain 85A was linked to a gene (denoted \textit{FCY} in Table 1) which underwent de novo mutation (\textit{FCY/FCY} → \textit{FCY/fcy}) to generate a resistance marker which failed to complement the \textit{fcy} alleles borne by the other parents of hybrids H1 through H6. The DNA content of one histidine-requiring resistant variant (72.1 fg per cell; not shown in Fig. 1) indicated that the variant arose (from hybrid H1) by a mechanism which did not result in a decrease in ploidy.
DISCUSSION

The present study was aimed at developing a cyclic parasexual system for C. albicans that is similar in principle to parasexual systems devised for other assexual fungi (7). We consider that this study provided the elements of such a system. Parent strains were mated by an established spheroplast fusion method to yield hybrids from which derivatives were obtained which were similar in ploidy to the parent strains. The latter result, reduction in ploidy, was an essential step in devising a cyclic system.

The parent strains were chosen to yield hybrids from which variants which resulted from the loss of at least one chromosome might be selected for increased resistance to 5-FC. It was considered possible that the loss of one chromosome would create an imbalance, in terms of the ratios of important gene products in the cell, which would result in selection for genomic balance attainable by further chromosome loss. Reduction in ploidy to some lower balanced state corresponding to the parental ploidy or to some aneuploid states might then be expected. However, it should be noted that the mechanisms of ploidy reduction remain to be investigated and that the validity of the foregoing rationale remains to be tested.

Spheroplast fusion by an established method readily yielded hybrids; these were selected by nutritional complementation and were found to display the expected 5-FC resistance phenotype. Hybridization was verified by comparison of the cellular DNA contents of the parents and hybrids. Our finding that the DNA content of one hybrid was unexpectedly high suggested that routine analysis of the DNA content of a hybrid is likely to be useful in future studies because genetic analysis in the absence of this information may yield confusing results. The literature contained only one similar confirmation of hybridization, that of Sarachek et al. (14), who found significant variation in DNA content among the hybrids obtained from a fusion cross. Confirmation by estimation of DNA content also serves to distinguish hybrids from revertants and thus permits effective hybridization experiments with only one auxotrophic marker per parent.

The present hybrids were expected to give rise to variants which displayed increased resistance to 5-FC, and such variants were readily obtained by means of a powerful selection method. Variants which are considered to have resulted from loss of chromosomes were readily obtained; they constituted the majority of variants in the present (nonrandom) sample, and our results suggest that they are likely to constitute a significant fraction of the variants in a random sample. In contrast, resistant variants which clearly resulted from loss of chromosomes were not obtained from two parent strains which were diploid or near-diploid. It remained possible that the parent strains gave rise to resistant variants due to loss of chromosomes but that other factors prevented isolation of variants which displayed a detectable decrease in ploidy. Clearly, the presence in the genome of recessive lethal alleles (let alleles) would render most (or all) such variants nonviable. Recessive lethal alleles were demonstrated in one C. albicans isolate (which was not among the strains studied here), and evidence for the occurrence of recessive lethal alleles in other isolates was presented (19). We should note, however, that the presence of let alleles would not prevent isolation of diploid (or triploid or aneuploid) variants from hybrids because these variants may carry let alleles in the masked (LET/let) state.

The present study provided preliminary linkage data for three genes. Use of the instability in ploidy observed in the present study as a mapping method may be expected to generate a linkage map for this species. Further studies are necessary to test the generality of ploidy instability in C. albicans. We shall show elsewhere that instability in ploidy was induced in hybrids by low doses of ultraviolet radiation (Whelan and Markie, in press). Determination of cell volume may provide a convenient criterion for recognition of useful variants derived from hybrids.

In idealized form, the suggested parasexual cycle may be written: 2n → 2n−4n → 2n. The present study suggests that such a cycle is attainable but that reduction in ploidy is a complex process and that extensive biochemical and genetic characterization of parents, hybrids, and variants is necessary.

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LITERATURE CITED