Plant Genetic Archaeology: Whole-Genome Sequencing Reveals the Pedigree of a Classical Trisomic Line

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ABSTRACT The circadian oscillator is astonishingly robust to changes in the environment but also to genomic changes that alter the copy number of its components through genome duplication, gene duplication, and homeologous gene loss. While studying the potential effect of aneuploidy on the Arabidopsis thaliana circadian clock, we discovered that a line thought to be trisomic for chromosome 3 also bears the gi-1 mutation, resulting in a shorter period and late flowering. With the help of whole-genome sequencing, we uncovered the unexpected complexity of this trisomic stock’s history, as its genome shows evidence of past outcrossing with another A. thaliana accession. Our study indicates that although historical aneuploidy lines exist and are available, it might be safer to generate new individuals and confirm their genomes and karyotypes by sequencing.

KEYWORDS
trisomic line  circadian period flowering time  GIGANTEA deep-sequencing

The anticipation of daily transitions like dawn and dusk by a circadian clock provides a fitness advantage in cyanobacteria, plants, fungi, and animals (Dodd et al. 2005; Green et al. 2002; Xu et al. 2011; Yerushalmi et al. 2011). Although the circadian clock comprises a highly interconnected network of transcriptional activators and repressors, this adaptation to a rhythmic environment has been maintained while the expansion of gene families and whole-genome duplication or triplication events have altered the balance between different clock components. With the advent of genome-sequencing platforms, we are now in an ideal position to study the evolutionary trajectory of clock gene families, including gene copy number variation, and the birth and death of new copies. Because of its network properties, the circadian clock constitutes a great model system in which to test the gene balance hypothesis. Indeed, Albert Blakeslee observed already in the early 1920s that plants with three copies of a single chromosome are generally less vigorous and fertile than polyploid lines in which all chromosomes have been triplicated, leading to the formulation of genetic (or gene) balance (Blakeslee et al. 1920; Blakeslee 1921). In Arabidopsis thaliana, some circadian mutants like the toc1-1 allele were first thought to exhibit dosage sensitivity (Millar et al. 1995), but this was later attributed to a dominant-negative mutation in the repressor domain of the protein (Gendron et al. 2012). Most A. thaliana clock mutations have recessive effects, indicating that a single gene copy is sufficient to maintain period length close to 24 hr, and thus demonstrates some dosage compensation. However, increasing gene copy number for TOC1 (Mas et al. 2003) or ZTL (Somers et al. 2004) changes period length in a dose-dependent manner, suggesting that network stoichiometry is important. A related phenomenon has been observed in mice, where segmental trisomy 16 shortens the clock period in constant darkness, demonstrating that aneuploidy may lead to alterations in circadian rhythms (Ruby et al. 2010). Following whole-genome duplication, clock genes in Brassica rapa are more likely to be retained compared with their neighboring genes or random and control genes (Lou et al. 2012). Properly functioning circadian clocks confer fitness benefits (Dodd et al. 2005; Green et al. 2002), putting strong selective pressure on clock genes to be maintained in a stoichiometrically balanced fashion. We wished to determine what circadian phenotypes might be observed in trisomic lines generated by George Redeí and others in the 1960s and available from the Arabidopsis stock centers (Redei 1962; Steinizt-Sears 1963). Although a stock presumed to be trisomic for chromosome 3, CS3227/N3227, had a shorter period, the cause was found to be a 5-bp deletion in the GIGANTEA gene found also in one of Redeí’s other stocks, gi-1 (Redei 1962; Park et al. 1999). Whole-genome sequencing revealed
that the supposedly trisomic stock is an introgression line, with one fifth of its genome coming from an Estland/Estonia (Est)-like accession. An accession from Est was one of ten Arabidopsis accessions widely studied by Friedrich Laibach (Laibach 1943) and later George Redeì (Redeì 1962), making it reasonable to deduce that this strain arose through inadvertent outcrossing.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

Seed stocks were ordered from stock centers: CS3227 [from Arabidopsis Biological Resource Center (ABRC)] and N3227 [from Nottingham Arabidopsis Stock Centre (NASC)], gi-1, gi-2, gi-3 (N3123, N3124, and N51 from NASC). The transfer DNA insertion allele gi-201 has been described (Martin-Tryon et al. 2007) and is presumed to be a null allele. Mutants ft-10 and flf-1 came from laboratory stocks. The TOC1:LUC reporter line in the Columbia-2 (Col-2) background was described previously (Salomé and McClung 2005). All experiments were performed at least three times (n = 12–24).

**Cotyledon movement**

Seeds were surface-sterilized by the vapor-phase method (Clough and Bent 1998) and plated on Murashige and Skoog (MS) medium supplemented with 2% sucrose and 0.8% agar. After stratification for 3 d at 4°C in the dark, plates were released and seedlings allowed to grow for 5 d under a photoperiod of 12-hr light: 12-hr dark, at 23°C. On day 6, individual seedlings were transferred to 24-well cloning plates on a cube of growth medium and released into constant light at 23°C. Cotyledon position was captured with surveillance cameras over 7 d. Postrun analysis was conducted with the help of the Kujata software package, and circadian parameters estimated by Fast-Fourier transform as previously described (Salomé and McClung 2005).

**Circadian analysis of LUC reporters**

Surface-sterilized seeds were plated on MS medium containing 1% sucrose and 0.5% agar and stratified for 2 d at 4°C in the dark. After release in 23°C, seedlings were entrained by light-dark cycles (16-hr light: 8-hr dark) for 7 d, and were then transferred to 96-well plates. Each well contained 200 µL of MS medium supplemented with 1% sucrose and 30 µL of 2.5 mM luciferin, potassium salt (Biosynth, Postfach, Switzerland). Plates were subjected to another entraining cycle and then moved into constant light and temperature for 5 d for luciferase activity measurements on a Perkin Elmer Topcount microplate luminescence counter. Circadian parameters were extracted from time-course data by Fast-Fourier transform as previously described (Salomé and McClung 2005). All experiments were performed at least three times (n = 12–24).

**Hypocotyl length measurements**

Surface-sterilized seeds were plated along the diagonal of Petri dishes containing full-strength MS medium supplemented with 1% sucrose and 0.8% agar. Plates were placed at 4°C for 3 d in the dark before being released in short days for 6 d. Images of plates were acquired on a flatbed scanner in transparent mode alongside a ruler. Hypocotyl length was measured with ImageJ (http://rsbweb.nih.gov/ij/).

| Table 1 Circadian parameters of genotypes |
|------------------------------------------|
| Genotype    | Period ± SEM | Amplitude ± SEM | RAEa ± SEM | n   |
|-------------|--------------|-----------------|-------------|-----|
| Col-0       | 24.2 ± 0.1   | 19,435 ± 1653   | 0.17 ± 0.01 | 24  |
| gi-1        | 21.9 ± 0.2   | 6222 ± 770      | 0.41 ± 0.03 | 23  |
| gi-2        | 25.0 ± 0.4   | 3154 ± 404      | 0.31 ± 0.03 | 22  |
| gi-201      | 24.1 ± 0.1   | 1878 ± 179      | 0.16 ± 0.02 | 12  |
| CS3227      | 21.2 ± 0.2   | 6111 ± 945      | 0.27 ± 0.02 | 12  |
| Col-0       | 24.3 ± 0.1   | 15,580 ± 1785   | 0.14 ± 0.01 | 23  |
| CS3227      | 22.0 ± 0.1   | 4909 ± 1004     | 0.20 ± 0.02 | 12  |
| lhy-20      | 21.6 ± 0.1   | 3628 ± 282      | 0.12 ± 0.01 | 12  |
| toc1-101    | 19.9 ± 0.1   | 980 ± 115       | 0.20 ± 0.03 | 23  |

a RAE refers to relative amplitude error, with an RAE of zero corresponding to a perfect sine wave, while RAEs closer to 1 denote weaker rhythms.
Next-generation sequencing and data analysis
Genomic DNA from gi-1, gi-2, Est-1 from the Max Planck Institute (MPI; CS2683), Est from the Salk Institute (CS67485), and CS3227 was isolated with the QIAGEN Plant Mini kit. A total of 1 μg of genomic DNA was used as starting material for Illumina’s TruSeq PCR-free library preparation according to manufacturer’s instructions for 350-bp insert size (Illumina, San Diego, CA). Final libraries were quantified by quantitative polymerase chain reaction (qPCR Kapa Biosystems, Boston, MA) and confirmed by Bioanalyzer (Agilent Technologies, Santa Clara, CA) and sequenced on a HiSeq2000 instrument with 2 × 101-bp reads. Short reads were processed with the SHORE pipeline (Schneeberger et al. 2009); after filtering of reads that did not map to the Col-0 reference genome, genome-wide coverage was about 20x for gi-1, 28x for gi-2, and 24x for CS3227, and more than 30x for Est accessions. High-quality single-nucleotide polymorphisms (SNPs) and small deletions between Col-0 and other genotypes were derived from shore consensus by the use of a scoring matrix optimized for identifying homoygous positions that differ from the reference genome (scoring_matrix_hom.txt). The resulting SNP lists were compared in R (The R Foundation for Statistical Computing 2013) with the help of the merge and intersect functions.

RESULTS
The genetic stock CS3227 has a short period
Seeds were obtained for a trisomic stock carrying three copies of chromosome 3 from the Arabidopsis Biological Resource Center. This stock, referred to herein as CS3227 (N3227 from NASC), displayed a short period when assayed by cotyledon movement (Figure 1A). This phenotype was observed in all seedlings from the original stock received from ABRC, as well as all subsequent generations derived from the original stock. This phenotype did not segregate in any of these populations (representing at least three generations of seed-to seed-propagation). Plants trisomic for chromosome 3 are expected to exhibit yellow-green rosette leaves and reduced fertility (www.arabidopsis.org), but we did not select progeny based on these phenotypes at any point, suggesting that the short circadian period was not linked to a compromised dosage of clock genes located on chromosome 3 but rather to an independent genetic defect that was fixed in this stock.

We introduced a TOC1::LUC luciferase reporter (in the Col-2 background; Solomé and McClung 2005) into CS3227 by genetic crossing. As shown in Figure 1 B and C, the short period phenotype segregated as a simple, recessive mendelian trait, further confirming that the circadian defect was not caused by aneuploidy. We selected several F2 seedlings with a short period and confirmed their phenotype in their progeny (Figure 1D and Table 1). Free-running period in CS3227 was 2 hr shorter than the stated parental line Col-2: 21.9 ± 0.1 hr for F3 lines (n = 12) vs. 24.2 ± 0.1 hr. CS3227 shortened period as much as lhy-20 (21.6 ± 0.1 hr, n = 12), a null allele of the LATE ELONGATED HYPOCOTYL gene, but remained longer than a strong allele of the TIMING of CAB2 1 gene (toc1-101: 19.7 ± 0.2 hr, n = 12; Figure 1D and Table 1).

The genetic stock CS3227 is late flowering
An additional phenotype, delayed flowering, was observed in CS3227 plants when grown in long days (16-hr light) at 23°C. The delay in initiation of the reproductive phase was similar to the other late flowering mutants fkJ1, ft, and gi (Figure 2 A and B). Late flowering and short period phenotypes of CS3227 always cosegregated (Figure

| Table 2 Frequencies of phenotypes and genotypes observed in a N3227 stock, obtained from NASC |
|---------------------------------|--------------|-------------|--------|
| Phenotype                      | Observed    | Expected * | % Observed |
| Late flowering                 | 106         | 29–30      | 100     | 0       |
|gi-1 / gi-1 genotype            | 106         | 29–30      | 100     | 0       |

NASC, Nottingham Arabidopsis Stock Centre.
* Transmission of the trisomic copy of chromosome 3 is based on Steinitz-Sears, 1963 (5 trisomics from 18 offspring, frequency = 27.8%, Steinitz-Sears 1963).
CS3227 behaves like gi-1 for circadian phenotypes

In light of the molecular lesion found in CS3227, we re-examined the circadian defects present in CS3227 alongside the weak gi allele gi-1 and the strong alleles gi-2 and gi-201. Both CS3227 and gi-1 displayed a similar short period, while the strong gi alleles did not affect period length significantly (Figure 3). Circadian amplitude of the reporter was markedly decreased in all gi mutants and in CS3227, with strong gi alleles having a stronger effect (with values about 10–14% of wild type, Figure 4). The same reporter was introgressed from Col-2 into all mutant backgrounds, allowing direct comparison of amplitudes. Again, gi-1 and CS3227 behaved in a similar fashion regarding circadian amplitude, which reached about 40% of wild-type values. The CS3227 genetic stock therefore exhibited all circadian defects known to occur in the gi-1 allele.

CS3227 differs from gi-1 for petiole and hypocotyl elongation

Not all aspects of CS3227 aligned with the phenotypes described for gi-1. GI loss of function alleles are characterized by elongated hypocotyls in red light and white light. We therefore measured hypocotyl length in Col-0, CS3227, and several gi alleles when grown in white light and short days. As expected, all gi alleles (gi-1, gi-2, and gi-201) had long hypocotyls under these conditions (mean length ~6 mm; with the Col-0 wild-type strain being about 3 mm in length) but remained slightly shorter than the red light photoreceptor mutant phyB-9 (Figure 5A). In contrast, CS3227 displayed a shortened hypocotyl that was comparable to Col-0. The long hypocotyl characteristic of a gi-1 mutant was recovered in an F2 population derived from a cross between CS3227 and Col-0, indicating that CS3227 also carried a modifier locus (or loci) that suppressed the long hypocotyl resulting from loss of GI function (Figure 5B). Petiole elongation is strongly promoted in gi mutants when grown in short days, as seen for gi-1 adult plants (Figure 5C). Petioles in CS3227 were much shorter, and were comparable to those seen in Col-0 (Figure 5C). We concluded that the CS3227 genetic stock carried both the gi-1 mutation (a 5-bp deletion) as well as a modifier locus (or loci) acting specifically in GI-dependent

![Figure 3](image1.png)

Comparison of CS3227 with other gi alleles for circadian period. All seedlings were grown under 12L:12D cycles for 7 d before being released into constant light to record circadian parameters. Mean normalized TOC1::LUC activity for Col-2 and CS3227 (A), gi-2 (B), and gi-1 (C) after release into constant conditions. (D) Mean TOC1::LUC period for seedlings shown in (A–C). All data shown as means ± SEM (n = 12–24).

![Figure 4](image2.png)

Comparison of CS3227 with other gi alleles for circadian amplitude. Mean TOC1::LUC amplitude for Col-2 and several gi alleles. Data are shown as box and whiskers plot from at least 24 seedlings.
control of cell elongation. These results also alleviate potential concerns about seed contamination or simple mislabeling of CS3227, as it clearly displayed a number of phenotypes not seen in gi-1.

Deep-sequencing of CS3227 reveals an unexpected pedigree
CS3227, gi-1, and gi-2 were isolated by George Redei in the early 1960s. That both CS3227 and gi-1 carry an identical 5-bp deletion raised the possibility of a shared genetic history of the two strains. We therefore sequenced the genomes of gi-1, gi-2, and CS3227 with the Illumina PCR-free DNA TruSeq protocol to identify polymorphisms relative to Col-0 in these genetic stocks and test for relatedness. SNP numbers relative to the Col-0 reference genome sequence are summarized in Table 3. As expected, gi-1 and gi-2 shared most of their SNPs, distributed along all 5 chromosomes. SNP numbers were low, consistent with the genetic background in which these two mutants were originally isolated (Col-1, which later gave rise to the commonly used Col-0 stock). A comparison with SNPs identified in CS3227 revealed a more complex picture. For much of the genome, CS3227 had 10–200 times more SNPs per chromosome than gi-1 or gi-2, but SNP density was distributed unevenly, with large-scale blocks of dense and sparse SNPs (Supporting Information, Figure S1, Figure S2, and Table 3). Chromosome 5 was an exception, with very few SNPs, many of which were shared with gi-1 and gi-2, presumably due to their common original background (Figure S1).

Although most Arabidopsis laboratories currently focus on one of the Col accessions or Ler, initial Arabidopsis research included 10 distinct accessions, all collected and distributed to the community by Friedrich Laibach (Laibach 1943). We therefore compared SNPs of a non-Col-0 portion of chromosome 1 from CS3227 with SNP datasets from seven of the nine non-Col accessions used by George Redei that have been re-sequenced as part of the 1001 Genomes project (http://1001genomes.org) (Weigel and Mott 2009) and found a match with Est (from Estland/Estonia; Figure S3). Blocks of high similarity between Est and CS3227 coincided with regions with high SNP density relative to Col-0, and were found on all chromosomes except chromosome 5 (Figure 6 and Figure S2).

Samples of the Est accessions have been sequenced at the MPI in Tübingen and at the Salk Institute, but only Est sequenced at the Salk Institute was a very good match to CS3227. We resequenced the Est accessions from Salk (CS67485) and from MPI (CS22683) with the PCR-free DNA TruSeq method used for our initial sequencing.

Table 3. Summary of SNP numbers identified in gi-1, gi-2, and CS3227 relative to the reference Col-0

| Genotype | chr 1   | chr 2   | chr 3   | chr 4   | chr 5   |
|----------|---------|---------|---------|---------|---------|
| gi-1     | 391     | 210     | 251     | 775     | 335     |
| gi-2     | 366     | 206     | 248     | 793     | 346     |
| CS3227   | 78,415  | 2506    | 8857    | 55,318  | 488     |

SNP, single-nucleotide polymorphism.

Figure 6 Estland/Estonia (Est) is a likely candidate progenitor for the non-Col-0 genomic regions found in CS3227. The Est accession shared most of its SNPs with the non-Col-0 regions of CS3227, here shown for chromosome 1 but also for chromosomes 2–4. (A) Inter-SNP distance for all SNPs found along chromosome 1 in CS3227 when Col-0 is used as reference. (B) Inter-SNP distance for chromosome 1 of CS3227 with Est as reference genome. (C) Complementary pattern of Col-0-like and Est-like genomic regions along chromosome 1 of CS3227. SNP, single-nucleotide polymorphism.
CS3227 was clearly related to Est from Salk, but not to Est-1 from MPI (Figure S3B). Both Est accessions displayed the lesioning/early senescence phenotype characteristic of accessions carrying a hyperactive allele of the ACCELERATED CELL DEATH 6 (ACD6) gene (Todesco et al. 2010). Of the two presumptive causal SNPs identified in the Est-1 allele of ACD6, only the polymorphism A566N was shared with the Est stock sequenced at the Salk Institute, suggesting that this SNP alone is sufficient to result in early onset senescence. CS3227 plants did not share this phenotype (Figure 2A); in agreement, the genomic location of ACD6 (about 8.3 Mb on chromosome 4) was of Col origin, although flanked by two Est regions (Figure S2).

Based on haplotype blocks, we identified clear breakpoints for all Est-like regions (Table 4). We counted several Est-like regions on chromosome 1 alone, indicative of at least eight independent crossover events resulting in the observed alternating pattern of Col-like and Est-like genomes. Because the average crossover rate for chromosome 1 is about 1.7 after a single meiosis (Salomé et al. 2012), this observation is consistent with an outcrossing event followed by many generations of selfing, leading to a pattern typical for recombinant inbred lines.

Finally, we wished to confirm that our CS3227 stock was no longer carrying a third copy of chromosome 3. We therefore plotted normalized read support for all identified Est-like SNPs for CS3227. As shown in Figure 7, read counts for CS3227 were even and showed a pattern very close to that seen in Est and CS3227. Importantly, normalized read count for all chromosomes was close to 1, including chromosome 3, indicating that any trisomic history in our stock had been lost prior to the generation we sequenced.

DISCUSSION

We initially set out to characterize what effect additional chromosomes might have on the Arabidopsis circadian clock. When it became clear that we did not have trisomic individuals in our test population, we shifted our attention to line CS3227, initially described as being trisomic for chromosome 3, which exhibited a number of interesting phenotypes rarely seen in combinations in other mixed genetic stocks, a non-negligible problem in arabidopsis (Anastasio et al. 2011). Although the study of changes in circadian parameters caused by aneuploidy merits further attention, it is clear that future efforts should begin with the generation, karyotyping, and phenotyping of aneuploid individuals from genetically defined parental lines. Chromosomal variants can now be easily identified by whole-genome sequencing (Henry et al. 2010), thus facilitating the precise identification of aneuploidy lines and the exact chromosome complement of their genomic make-up. It may also be informative to decrease clock gene copy number in a tetraploid background, to observe what possible circadian defects might arise from the loss of a clock gene following whole-genome duplication. Our study also highlights the power of whole genome sequencing to resolve issues arising from mixed genetic stocks, a non-negligible problem in Arabidopsis (Anastasio et al. 2011).

Whole-genome sequencing of the CS3227 genome revealed a mosaic of Col-like and Est-like regions for all but chromosome 5, arguing against a simple seed contamination (Figure 6, Figure S2, and Figure S3). We did not observe greater read coverage for chromosome 3 (Figure 7), consistent with a lack of trisomy in our population. A delay in flowering time was not reported for newly synthesized chromosome 3 trisomics (Henry et al. 2010), leading us to conclude (i) that this phenotype is specific for CS3227 and the gi-1 deletion, and (ii) that chromosome 3 does not contain genes with major roles in the switch to flowering. Indeed, very few genes with major effects on flowering time have been mapped to chromosome 3.

Even with full genome sequence from our CS3227 stock, it is difficult to reconstruct the exact chain of events that led to its genesis. Three major events must have taken place, the order of which is not clear: the isolation of a trisomic stock for chromosome 3; the introduction of the gi-1 deletion; and the introgression of an Est-like genome. Trisomic lines for other chromosomes do not share the gi-1 deletion (not shown), arguing against the model depicted in Figure 8B, in which the gi-1 mutant was used as diploid parent in the original diploid x tetraploid cross. gi-1 and the trisomic lines were both generated by irradiating A. thaliana with X rays (Redei 1962; Steinitz-Sears 1963); it is therefore conceivable that gi-1 was isolated in the progeny of the diploid x tetraploid cross (Figure 8, A and C). Although we favor this scenario, we notice that gi-1 and CS3227 do not share more SNPs with each other than with gi-2. However, ionizing agents do not induce as many point mutations as EMS (Belfield et al. 2012) and may therefore not live an easily trackable footprint.

The introduction of the Est-like genome in the ancestral CS3227 stock is more difficult to date. Because of the number of apparent crossovers, most notably on chromosome 1, many generations must separate the initial cross and the current generation we characterized and sequenced. Other trisomic lines were negative for an Est-like genotype at a small number of tested markers, suggesting that the induction of trisomy may have predated the introduction of the Est-like genome. The model depicted in Figure 8A is therefore the most likely scenario to explain how CS3227 came about.

Although the study of changes in circadian parameters caused by aneuploidy merits further attention, it is clear that future efforts should begin with the generation, karyotyping, and phenotyping of aneuploid individuals from genetically defined parental lines. Chromosomal variants can now be easily identified by whole-genome sequencing (Henry et al. 2010), thus facilitating the precise identification of aneuploidy lines and the exact chromosome complement of their genomic make-up. It may also be informative to decrease clock gene copy number in a tetraploid background, to observe what possible circadian defects might arise from the loss of a clock gene following whole-genome duplication. Our study also highlights the power of whole genome sequencing to resolve issues arising from mixed genetic stocks, a non-negligible problem in Arabidopsis (Anastasio et al. 2011).

**Table 4 Predicted Est-like intervals in CS3227**

| Chromosome | Left Border, bp | Right Border, bp |
|------------|----------------|-----------------|
| 1          | 10,180,611     | 19,875,136      |
|            | 21,543,989     | 22,196,062      |
|            | 28,423,595     | 30,427,017      |
| 2          | 0              | 302,692         |
| 3          | 1,732,545      | 4,274,768       |
|            | 4,289,162      | 4,622,728       |
| 4          | 0              | 6,215,838       |

Est, Estland/Estonia.
model (C), two diploid parents, Col-1 and Est-like, were crossed and may have been the diploid parent (B). The introduction of the Est-like segregation of chromosomes in the F1s was induced by X-ray irradiation mutagenesis of Arabidopsis thaliana. The nonequal (A) and (B), the complement of primary trisomics was generated from Bel.

Figure 8 Possible models to explain the genesis of CS3227. In models (A) and (B), the complement of primary trisomics was generated from a cross between a diploid and a tetraploid Col-1 parent. The nonequal segregation of chromosomes in the F1s was induced by X-ray irradiation mutagenesis of Arabidopsis thaliana. The introduction of the Est-like genome occurred after the isolation of trisomic individuals, either by targeted crossing or by outcrossing to a neighboring Est-like plant. In model (C), two diploid parents, Col-1 and Est-like, were crossed and the F1 plants X-ray irradiated. The extent of Est-like genome was subsequently largely lost following backcrosses to Col-1 or by single-seed descent. As in (A), model (C) posits the genesis of gi-1 as a consequence of X-ray irradiation of the F1 generation.

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