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Gene silencing in a polyploid homosporous fern: Paleopolyploidy revisited

(polyploidy/*Pellaea*/isozymes)

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ABSTRACT **Because of their high chromosome numbers, homosporous vascular plants were considered paleopolyploids until recent enzyme electrophoretic studies rejected this hypothesis by showing that they express only diploid numbers of isozymes. In polyploid sporophytes of the homosporous fern *Pellaea rufa*, however, progressive diminution of phosphoglucoisomerase activities encoded by one ancestral genome culminates in tetraploid plants exhibiting a completely diploidized electrophoretic phenotype for this enzyme. The demonstration that such gene silencing can make a polyploid fern look isozymically like a diploid questions the validity of isozyme evidence for testing the paleopolyploid hypothesis and supports the proposed role of polyploidization followed by genetic diploidization in the evolutionary history of homosporous pteridophytes.**

The homosporous ferns and other homosporous pteridophytes have long been regarded as the prime examples of polyploidy among the vascular plants because of their high chromosome numbers (mean n = 57.05) relative to those of heterosporous plants such as the angiosperms (mean n = 15.99) (1). The leading hypothesis to explain these high numbers attributes them to polyploidization in the ancestry of homosporous plants (paleopolyploidy) (2). Enzyme electrophoretic studies discussed below, however, have shown that the number of isozymes per enzyme in homosporous fern species with the lowest chromosome numbers in their genera is the same as in diploid angiosperms, implying that these ferns have a diploid number of gene loci encoding these enzymes. Because homosporous ferns do not express extra sets of genes as expected in polyploids, these results have discredited the hypothesis that their high chromosome numbers result from paleopolyploidy. A revised version of the paleopolyploid hypothesis (3) reconciles these data by proposing that fern phylogeny has involved repeated cycles of polyploidization followed by genetic diploidization involving gene silencing. This proposal accounts for the lack of isozyme evidence for paleopolyploid loci by asserting that the extra loci have been silenced, an assertion that is speculative inasmuch as no direct evidence of gene silencing in ferns has been published to date. This report provides direct evidence of diploidization of electrophoretic phenotypes in a polyploid homosporous fern and thus supports the view that polyploidization coupled with genetic diploidization could be an important evolutionary mechanism in this group.

MATERIALS AND METHODS

The four populations of South African *Pellaea rufa* A. F. Tryon used in this study, voucher deposition, and electrophoretic protocols are as described (4), except that zymograms in Fig. 1 *B–D* used gel/electrode system 7 and those in Fig. 1 *A* and *E–H* used system 8 in ref. 5. This study is based on phosphoglucoisomerase (PGI; EC 5.3.1.9), a particularly informative dimeric enzyme in which formation of heterodimers facilitates interpretation of critical genotypes. PGI exhibits cytosolic and chloroplastic isozymes (6, 7), but in *P. rufa* the chloroplastic isozyme (1 in Fig. 1 *A* and *E*) migrates anodally to the cytosolic isozymes (2 and 3 in Fig. 1 *A* and *E*) and does not resolve into bands. Thus, chloroplastic PGI bands do not complicate interpreting the banding patterns of the cytosolic isozymes. Allozymes of PGI are labeled with the most anodally migrating designated A, the next most anodal labeled B, etc., and with the corresponding coding alleles designated *a*, *b*, etc. *P. rufa* (n = 58 chromosome pairs) is tetraploid (4) relative to its congeners (most of which have n = 29 pairs) regardless of the paleopolyploid hypothesis.

RESULTS

P. rufa is an allotetraploid species combining two ancestral genomes, one contributing alleles *a* and *b* at the gene locus coding cytosolic PGI and the other contributing alleles *c* and *d* at this locus (4). Tetraploid sporophytes heterozygous for these alleles in both ancestral genomes exhibit a crowded 10-banded pattern (Fig. 1*A*) consisting of four homodimers (AA, BB, CC, DD), two intragenomic heterodimers (AB, CD), and four intergenomic heterodimers (AC, AD, BC, BD). When subjected to electrophoresis, individual gametophytes meiotically derived from such doubly heterozygous sporophytes exhibit one of four three-banded patterns (Fig. 1*B*) consisting of one homodimeric band specified by each ancestral genome plus an intergenomic heterodimer. The cytosolic isozyme of PGI encoded by the *ab* ancestral genome is designated PGI2 (2 in Fig. 1 *A* and *E*), and its coding gene is *Pgi2* with allelic variants $Pgi2^a$ and $Pgi2^b$. The isozyme encoded by the *cd* ancestral genome is designated PGI3 (3 in Fig. 1 *A* and *E*), and its coding gene is *Pgi3* with allelic variants $Pgi3^c$ and $Pgi3^d$. Even sporophytes homozygous at *Pgi2* and *Pgi3* beget gametophytes with a three-banded cytosolic PGI pattern, because each gametophyte receives one ancestral chromosome set containing *Pgi2* and one containing *Pgi3*.

Exceptional sporophytes did not produce *Pgi3*-encoded bands, and exceptional gametophytes failed to produce either *Pgi3*-encoded homodimers or both those homodimers and the intergenomic heterodimers. It was also observed that the PGI3 isozyme is often less active in gels than is PGI2. In Fig. 1*C*, for example, although *Pgi3* allozyme DD is highly active in the sporophyte in the last lane, a considerable reduction in activity of *Pgi3* allozymes CC and DD is seen in the other lanes. These observations suggested a defect in the pathway between transcription of *Pgi3* and protein activity. The

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Abbreviation: PGI, phosphoglucoisomerase.

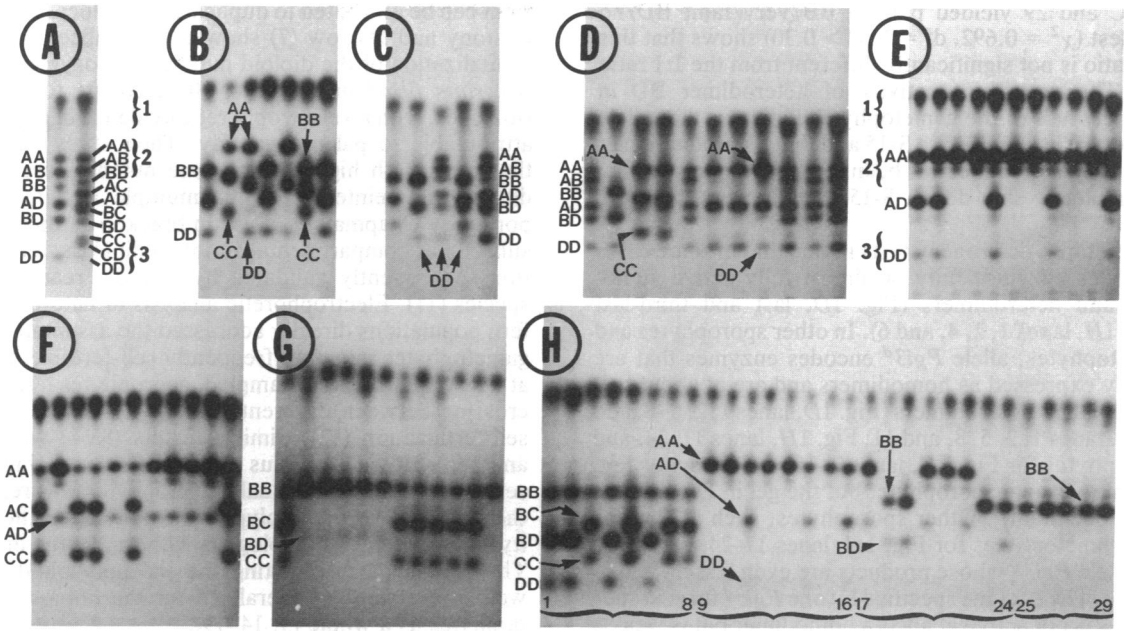

FIG. 1. Zymograms showing electrophoretic PGI banding patterns of *P. rufa*, with anode toward top of each zymogram. (*A*) Right lane interprets the complex pattern of four homodimeric and six heterodimeric bands in tetraploid sporophyte S-14 heterozygous at *Pgi2* and *Pgi3* encoding cytosolic isozymes 2 and 3, respectively; left lane contrasts the simpler pattern of sporophyte S-4 homozygous for allele *d* at *Pgi3*. Bands AD and BC are contiguous and partially overlapping, and bands CC and CD are contiguous. (*B*) Segregation of homodimeric PGI2/PGI3 phenotypes AA/CC, AA/DD, BB/CC, and BB/DD (with respective unlabeled heterodimeric bands AC, AD, BC, and BD) in gametophytes meiotically derived from a sporophyte with the phenotype shown in lane 2 of zymogram *A*. (*C* and *D*) Selected sporophytic phenotypes from the four study populations, showing varying degrees of expression of the *cd* genome. (*E–G*) Segregation patterns of randomly selected single gametophytes meiotically derived from respective sporophytes. (*E*) Eleven gametophytes from sporophyte B-21 whose PGI phenotype is shown in lane 8 of zymogram *D*. (*F*) Eleven gametophytes from sporophyte B-4 whose PGI phenotype is shown in lane 3 of zymogram *D*. (*G*) Twelve gametophytes from sporophyte S-15 whose PGI phenotype is shown in lane 1 of zymogram *C*. (*H*) Single gametophyte progenies illustrating the full range of expression of allele $Pgi3^d$ in homodimeric and heterodimeric bands. Gametophytes used in lanes 1–8 are from sporophyte B-15 whose PGI phenotype is not illustrated; gametophytes used in lanes 9–16 are from sporophyte B-21 whose PGI phenotype is shown in lane 8 of zymogram *D* (the patterns in lanes 9–16 are the same as in zymogram *E* except that zymogram *E* was overstained to accentuate allozyme DD); gametophytes used in lanes 17–24 are from sporophyte B-1 whose PGI phenotype is not illustrated; gametophytes used in lanes 25–29 are from sporophyte B-2 whose PGI phenotype is not illustrated but would be indistinguishable from the patterns in lanes 25–29.

genetic basis of these exceptional phenotypes was investigated by electrophoresing individual gametophytes meiotically derived from sporophytes with informative banding patterns. Several examples illustrate the range of results obtained.

Sporophyte B-21 (Fig. 1*D*, lane 8) displays homodimeric bands AA and DD plus heterodimeric band AD, suggesting that its genotype for cytosolic PGI is $Pgi2^a2^a3^d3^d$. With this genotype, sporophyte B-21 would have no intragenomic heterozygosity, and meiosis could deliver to each gametophyte only one dose of allele $Pgi2^a$ and one dose of allele $Pgi3^d$. Each gametophyte should therefore display the same three-banded cytosolic PGI phenotype as its parental sporophyte. Electrophoresis of 144 single gametophytes from this sporophyte (11 are seen in Fig. 1*E*) yielded 77 with expected bands AA/AD/DD, but the remaining 67 gametophytes unexpectedly displayed only band AA, with no band DD and no AD heterodimer even when the gel was overstained as in Fig. 1*E*. Thus the sporophytic pattern is not fixed in its derivative gametophytes. These data are explained if the sporophyte's genotype for cytosolic PGI was not $Pgi2^a2^a3^d3^d$ but rather $Pgi2^a2^a3^d3^{null}$. In that case, half of B-21's gametophyte progeny should receive alleles $Pgi2^a$ and $Pgi3^d$, yielding the observed AA/AD/DD phenotype, and half should receive alleles $Pgi2^a$ and $Pgi3^{null}$, yielding observed phenotype AA. A χ^2 test ($\chi^2 = 0.694$, df = 1, $P > 0.30$) shows that the observed ratio of gametophyte phenotypes (77:67) is not statistically different from the 1:1 ratio expected under the hypothesis that there is a null allele at *Pgi3*.

Sporophyte B-4 (Fig. 1*D*, lane 3) exhibits highly active bands AA/AC/CC, very weak heterodimeric band AD (cathodally contiguous with AC), but no band DD. These data are explained if sporophyte B-4 has genotype $Pgi2^a2^a3^c3^{d\sim null}$ for cytosolic PGI, where "$\sim null$" means "nearly null." In this case, the enzyme product of $Pgi3^{d\sim null}$ is not active in homodimeric form but is weakly active in heterodimeric form. If the hypothesized sporophytic genotype $Pgi2^a2^a3^c3^{d\sim null}$ is correct, individual gametophytes should segregate 1:1 for genotypes $Pgi2^a3^c$ and $Pgi2^a3^{d\sim null}$ that will be electrophoretically visualized as gametophytic banding patterns AA/AC/CC and AA/faint AD/no DD, respectively. When 60 randomly selected individual gametophytes from this sporophyte were subjected to electrophoresis (11 are seen in Fig. 1*F*), 28 displayed banding pattern AA/AC/CC and 32 displayed the pattern AA/faint AD/no DD. A χ^2 test ($\chi^2 = 0.266$, df = 1, $P > 0.50$) shows that this is not significantly different from the 1:1 ratio that is expected.

Sporophyte S-15 (Fig. 1*C*, lane 1) clearly presents bands BB/BC/CC, with CC considerably more faint than BB. These staining intensities cannot be attributed to a tetraploid sporophytic genotype with three doses of allele $Pgi2^b$ and one dose of allele $Pgi3^c$, because alleles *b* and *c* are at different ancestral loci (*Pgi2* and *Pgi3*, respectively), and the tetraploid sporophyte should have two copies of *Pgi2* and two copies of *Pgi3*. Instead, it was hypothesized that band CC is faint because it is encoded by only a single dose of allele $Pgi3^c$, with the remaining allele at *Pgi3* being null or nearly null. This would be true if sporophyte S-15's genotype is either $Pgi2^b2^b3^c3^{c=null}$ or $Pgi2^b2^b3^c3^{d=null}$. When 52 single gametophytes from this sporophyte were subjected to electrophoresis (12 are seen in Fig. 1*G*), 23 yielded banding pattern

BB/BC/CC and 29 yielded pattern BB/very faint BD/no DD. A χ^2 test ($\chi^2 = 0.692$, df = 1, $P > 0.30$) shows that this observed ratio is not significantly different from the 1:1 ratio expected. The very faint activity of heterodimer BD in approximately half of the gametophytes indicates that allele $Pgi3^d$ is present in sporophyte S-15 and that its production of functional enzyme product has been strongly diminished but not yet completely shut down. S-15's genotype is therefore $Pgi2^b2^b3^c3^{d\sim null}$.

Thus gametophytic progeny tests reveal that in some cases $Pgi3^d$ encodes enzymes fully or almost fully active as homodimers and heterodimers (Fig. 1*D*, last and third-last lanes; Fig. 1*H*, lanes 1, 2, 4, and 6). In other sporophytes and their gametophytes, allele $Pgi3^d$ encodes enzymes that are more faintly expressed as homodimers and are at about half normal strength as heterodimers [Fig. 1*D*, lanes 1, 2, 5–8, and 10; Fig. 1*E*, lanes 1, 2, 5, 8, and 11; Fig. 1*H*, lanes 11, 14, and 16 (gametophytes for Fig. 1*H*, lanes 9–16, and those for Fig. 1*E* are from the same sporophyte, but the gel in Fig. 1*E* was allowed to overstain)]. Other sporophytes, such as the one providing gametophytes for Fig. 1*H*, lanes 17–24, carry two forms of allele $Pgi3^d$ whose products are even less enzymatically active. The enzyme specified by one $Pgi3^d$ form in such sporophytes is not active at all as a homodimer but is weakly active as a heterodimer (Fig. 1*H*, lanes 17, 20, and 22, where D is heterodimeric with A; lanes 18, 19, and 24, where D is heterodimeric with B). The enzyme encoded by the other $Pgi3^d$ form from this same sporophyte is not active in homodimeric or heterodimeric form regardless of whether the gametophyte carrying it also carries allele $Pgi2^a$ or allele $Pgi2^b$ (Fig. 1*H*, lanes 21 and 23). Finally, in still other sporophytes, both copies of allele $Pgi3^d$ are completely silenced with regard to functional enzyme expression under standard assay conditions, so that the sporophyte and all of its derivative gametophytes are single-banded for cytosolic PGI (Fig. 1*H*, lanes 25–29). This latter case is phenotypically indistinguishable from the single-banded pattern for cytosolic PGI that is observed in a diploid sporophyte homozygous at this locus (for example, in diploid *Pellaea andromedifolia*, lanes 7, 8, 10, and 11 of figure 6 in ref. 4). Tetraploid sporophytes of this latter type have therefore been completely genetically diploidized with regard to cytosolic PGI.

DISCUSSION

Homosporous ferns are characterized by bisexual gametophytes capable of self-fertilization that results in instant homozygosity at all loci on homologous chromosomes (homologous homozygosity). Assuming that their high chromosome numbers result from paleopolyploidy that yields extra (homoeologous) chromosome sets, it was hypothesized that genetic variation gradually accumulated in the homoeologous genomes (homoeologous heterozygosity) would compensate for homologous homozygosity resulting from putatively frequent self-fertilization in nature (1, 8). Multiple enzyme bands observed in electrophoresis of bracken fern (*Pteridium aquilinum*) sporophytes were proposed as evidence that ferns do indeed have multiple copies of genes on homoeologous chromosome sets resulting from paleopolyploidy (9).

Subsequent enzyme electrophoretic analyses of homosporous ferns discredited the evidence from bracken and the hypothesis of paleopolyploidy. Studies of meiotic segregation expressed in gametophytes (10) demonstrated that at least some multibanded enzyme patterns in sporophytes result from normally segregating diploid Mendelian heterozygosity, explaining some of the multiple bands reported by Chapman *et al.* (9) in bracken. Gottlieb (6) showed that even plants with diploid genomes have multiple subcellularly compartmentalized isozymes for certain enzymes and that only multiple isozymes located in a single subcellular compartment can be attributed to duplicated gene loci. Based on this, Gastony and Darrow (7) showed that subcellular compartmentalization of the diploid number of isozymes in the fern *Athyrium filix-femina* ($2n = 80$) accounts for its multiple isozymes, with no electrophoretic evidence of duplicated loci attributable to paleopolyploidy. Their study demonstrated that ferns with high chromosome numbers are genetically diploid and reinterpreted the multiple isozyme bands reported by Chapman *et al.* (9) in bracken as those typical of subcellular compartmentalization in diploids, a reinterpretation subsequently validated by a direct reanalysis of this species (11). Electrophoretic analysis of naturally occurring fern populations directly addressed the assumption that fern gametophytes in nature frequently self-fertilize, finding that at least 81% of the sampled sporophytes arose through crossing between different gametophytes and not through self-fertilization (12). Similar results (83%) were found in another study (13). Thus the hypothesized high level of self-fertilization in bisexual gametophytes was rejected, and the supposed need for polyploid homoeologous heterozygosity to counteract homologous homozygosity disappeared. These findings discrediting the paleopolyploid hypothesis were subsequently generalized for the homosporous pteridophytes as a whole (3, 14–19).

Nevertheless, the origin and significance of high chromosome numbers in homosporous vascular plants remain controversial. The hypothesis that vascular plants may have originated with high chromosome numbers cannot be rejected (20), but it is not generally accepted (2, 16). The competing recently revised hypothesis of paleopolyploidy (3) proposes that the high chromosome numbers of homosporous ferns result from an evolutionarily upward spiral involving repeated cycles of polyploidization followed by chromosomal diploidization and gene silencing. The resulting neodiploids would have high chromosome numbers that pair as bivalents at meiosis and express only diploid isozyme patterns. Circumstantial evidence for silencing of duplicated genes in allopolyploid fern species has been proposed (21), as has a model for speciation in polyploid pteridophytes based on reciprocal silencing of homoeologous genes (22). Preliminary data supporting silenced gene expression in tetraploid ferns have been discussed (23, 24), and multiple copies of defective chlorophyll a/b-binding protein genes that may result from gene silencing following polyploidy in *Polystichum munitum* have been reported (25). However, although direct genetic evidence of silenced duplicated genes encoding isozymes has been provided in angiosperms (26–28), no comparable evidence has previously been published for polyploid ferns. This report provides direct genetic evidence that progressive silencing of the gene for cytosolic PGI in one ancestral genome of tetraploid *P. rufa* results in a completely diploidized electrophoretic phenotype for that enzyme.

Loss of homodimeric PGI3 activity and diminution or loss of heterodimeric PGI3 activity in *P. rufa* is similar to that reported for cytosolic PGI in the angiosperm *Clarkia xantiana* (29). In that species, reduced heterodimeric PGI activities in plants that did not display one of the two homodimeric bands suggested that although mutant subunits were probably full length, they did not form stable dimers with normal subunits or with each other. Another study of *Clarkia* showed that inability to visualize an isozyme electrophoretically need not indicate inactivity of that isozyme *in vivo*, but it also showed a correlation between inactivity in gel assays and reduction of activity *in vivo* as measured spectrophotometrically (30). Degree of PGI3 activity *in vivo* has not been determined for *P. rufa* plants totally null for activity of this isozyme in gels, but this is irrelevant to the relationship between paleopolyploidy and diploidization of zymogram patterns. Overall levels of cytosolic PGI activity are the same in species of *Clarkia* with and without duplication (tetra-

ploidization) of the cytosolic PGI locus, indicating that some form of regulation has evolved that compensates for the duplicated genes (31). The *Clarkia* study suggested that gene dosage compensation depends on factors that regulate levels of transcription and translation.

In *P. rufa*, variably diminished PGI3 activity may result from regulation of transcription or translation or from mutation of the structural gene itself. If little monomer is produced as a result of down-regulation of transcription or translation, most of it will combine with the undiminished supply of monomer from normally functioning *Pgi2* and therefore most of the diminished supply of *Pgi3* monomers will be visualized in heterodimeric form. That could explain why homodimers disappear before heterodimers in nearly null forms of $Pgi3^d$. Depending on the amount of transcript translated, the bands coded by *Pgi3* would show the full range of intensity illustrated in the examples above. Alternatively, variably diminished *Pgi3* expression may result from mutation of the structural gene itself. In this case, variant forms of *Pgi3* would be transcribed and the transcripts would be translated into variously modified monomers (possibly including monomers of incomplete length) yielding more or less variant dimeric quaternary protein structures or unstable dimers as suggested in the *Clarkia* study above. Depending on the severity of mutation in the structural gene, this would reduce or completely nullify the protein's ability to act enzymatically in homodimeric form or in both homodimeric and heterodimeric form.

Whatever the mechanism responsible for diminished activity of the *Pgi3*-encoded isozyme in *P. rufa*, the result is a gene that has been effectively silenced, with several stages in the silencing still extant and detectable in these natural populations. Although silencing is more complete for $Pgi3^d$ than $Pgi3^c$, all cytosolic PGIs specified by the *cd* ancestral genome are usually enzymatically weaker in gels than those encoded by the *ab* ancestral genome, suggesting that incipient silencing affects the *Pgi3* locus in general and that, if extended to other loci, this mechanism could eventually lead to full genetic diploidization of this tetraploid species. However, even if this were simply a case of silencing an isolated gene, it demonstrates the reality of genetic diploidization in a polyploid fern and to that extent supports genetic diploidization as a credible mechanism in pteridophyte evolution.

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