The Maternally Expressed WRKY Transcription Factor TTG2 Controls Lethality in Interploidy Crosses of Arabidopsis

Brian P. Dilkes1,2, Melissa Spielman3, Renate Weizbauer2, Brian Watson2, Diana Burkart-Waco1, Rod J. Scott3, Luca Comai1,2*

1 Section of Plant Biology and Genome Center, University of California Davis, Davis, California, United States of America, 2 Department of Biology, University of Washington, Seattle, Washington, United States of America, 3 Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom

The molecular mechanisms underlying lethality of F1 hybrids between diverged parents are one target of speciation research. Crosses between diploid and tetraploid individuals of the same genotype can result in F1 lethality, and this dosage-sensitive incompatibility plays a role in polyploid speciation. We have identified variation in F1 lethality in interploidy crosses of Arabidopsis thaliana and determined the genetic architecture of the maternally expressed variation via QTL mapping. A single large-effect QTL, DR. STRANGELOVE 1 (DSL1), was identified as well as two QTL with epistatic relationships to DSL1. DSL1 affects the rate of postzygotic lethality via expression in the maternal sporophyte. Fine mapping placed DSL1 in an interval encoding the maternal effect transcription factor TTG2. Maternal parents carrying loss-of-function mutations in TTG2 suppressed the F1 lethality caused by paternal excess interploidy crosses. The frequency of cellularization in the endosperm was similarly affected by both natural variation and ttg2 loss-of-function mutants. The simple genetic basis of the natural variation and effects of single-gene mutations suggests that F1 lethality in polyploids could evolve rapidly. Furthermore, the role of the sporophytically active TTG2 gene in interploidy crosses indicates that the developmental programming of the mother regulates the viability of interploidy hybrid offspring.

Introduction

Postzygotic lethality of the hybrid offspring from crosses between divergent parents functions as a barrier that drives or reinforces speciation [1–16]. The molecular mechanisms underlying this response, however, are not understood. In plants, an example of F1 lethality occurs when progeny from crosses between newly formed polyploid individuals and their diploid progenitors fail during seed development. This lethality arises in the absence of any allelic diversity due to the difference in the dosage of genomes provided by the parents. The strength of this reproductive isolation can vary within species and between individuals of the same species, and has long been known to contribute to speciation. Yet, we understand neither the genetic basis of this barrier nor the source of variation within and between species.

Fertilizations between haploid and diploid gametes can occur in diploid species due to meiotic errors that produce unreduced (2n) gametes. In humans, for example, triploidy is associated with spontaneous miscarriage, causing postzygotic lethality in approximately 2% of all conceptions between parents of apparently normal karyotypes [17]. In diploid plants, the frequency of unreduced gametes can approach a few percent depending on the species, genotype, and environmental conditions [18–20]. Triploids from the union of normal and unreduced gametes can form tetraploid plants. Yet, the rates of new tetraploid formation are often orders of magnitude lower than unreduced gamete formation [9,21]. Still, the frequency of polyploid formation is greater than typical base substitution rates, such as estimated for Arabidopsis at approximately 1/108 [22].

Postzygotic lethality in interploidy crosses is hypothesized to play an important role in polyploid speciation by genetically isolating populations of divergent ploidy [1–16]. Polyploid speciation is very common in the flowering plants, in which it is phylogenetically coincident with 4%–10% of all plant speciation events [21]. In addition to recent polyploid speciation events, whole-genome duplication from ancient polyploidy events is a feature detected in many plant, vertebrate, fungal, and protist genomes [23–39]. Thus, the establishment of polyploid lineages and polyploid speciation has played a role in the evolution of all multicellular eukaryotic lineages.


Academic Editor: Detlef Weigel, Max Planck Institute for Developmental Biology, Germany

Received August 20, 2008; Accepted October 29, 2008; Published December 9, 2008

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Abbreviations: BC, backcross; CSS, chromosome substitution strain; DAP, days after pollination; DSL1, DR. STRANGELOVE 1; EOP EPISTATIS OVERCOMES PATERNAL EXCESS; FIST FERTILIZATION INDEPENDENT SEED2; RIL, recombinant inbred line; SNP, single nucleotide polymorphism; STAIRS, aligned inbred recombinant strain; TTG2, TRANSPARENT TESTA GLABRA2

* To whom correspondence should be addressed. E-mail: lcomai@ucdavis.edu

† Current address: Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, Indiana, United States of America.
Author Summary

Many flowering plants tolerate changes in the number of genome copies (ploidy), but offspring of parents with different ploidies often fail to develop. We investigated this phenomenon in Arabidopsis thaliana and discovered variation in the ability to survive interploidy matings. Two common strains (known as accessions), Ler and Col, are respectively permissive and intolerant when diploid females are mated to tetraploid males of Col. We mapped the genes responsible for this response and identified a major locus, which we call DR. STRANGELOVE1, on chromosome 2, and after finer mapping, defined the locus TRANSPARENT TESTA GLABRA2 (TTG2) as the candidate gene. TTG2 regulates the fate of interploidy crosses, and knock-outs of TTG2 improved the outcome of interploidy matings in both Ler and Col. Furthermore, the two accessions differed in genotype and in expression of this gene. TTG2 is an epidermal regulator whose activity affects seed endosperm development. Importantly, TTG2 acts within tissue of the seed-plant, indicating that a transgenerational interaction is responsible for controlling the outcome of interploidy mating.

Complete reproductive isolation is not always achieved following a shift in ploidy, and the penetrance of the interploidy hybridization barrier varies within and between species [9,40,41]. In species without strong postzygotic lethality in interploidy crosses, polyploid derivatives can be formed via the fusion of a single unreduced gamete with a normal haploid gamete. When the resulting triploids are fertile, they can give rise to triploid and diploid offspring via the so-called triploid bridge [9]. In some plants, including Arabidopsis thaliana [42], very little postzygotic lethality has been observed in crosses between individuals of divergent ploidy, and the generation of tetraploids via a triploid bridge has been demonstrated [5,9,43,44]. Although this may increase the rate of polyploid formation, the recurrent formation of fertile triploids from interploidy crosses between tetraploid and diploid populations would permit the sharing of alleles between the two cytotypes and hinder polyploid speciation.

Work with karyotypic variants and interspecies hybrids by Nishiyama and Yabuno [45], Johnston et al. [16], and Lin [46] demonstrated that the contributions of the pollen and seed parents are unequal and that a balance between these contributions is required for normal sexual reproduction in plants. In interploidy hybrids, doubling the genome of only one parent upsets this balance and can result in seed lethality. Remarkably, species vary in the combining valence of their genomes such that some interspecies crosses are more successful when individuals of divergent ploidy are crossed. The term endosperm balance number (EBN) was proposed to describe the pattern of dosage-dependent incompatibility intrinsic to each species or accession [16]. Such phenomena are not limited to angiosperms. Vertebrate interspecies hybridizations, such as crosses within the genus Hyla [47], display increased fertility when the maternal parent is in genomic excess. The interploidy barrier is also affected by the direction of the cross, with maternal excess crosses, e.g., 4x × 2x, typically resulting in less postzygotic lethality than paternal excess (2x × 4x) crosses [9]. A similar effect is typically seen for postzygotic F1 lethality in plant species barriers, including the lethality found in crosses between A. thaliana and A. arenosa, in which increasing the relative maternal dosage increases seed survival [48,49].

The similarity of progeny phenotypes from interploidy and interspecies crosses, and the shared sensitivity to changes in genome dosage, has led a number of authors to hypothesize that both forms of F1 lethality could be controlled by the same mechanisms [16,49–52]. One expectation has been that variation in imprinted genes, in which expression in the offspring depends on the parental origin of the chromosome, is responsible for the postzygotic lethality observed in interploidy crosses [46,52–57]. As both interploidy and interspecies hybridization barriers are affected by genome dosage, multiple dosage-sensitive genetic mechanisms, inclusive of imprinting, have been proposed [16,45,51,52,56,58]. The differential dosage hypothesis [51] attempts to unify these various proposals by postulating that any differential parental contribution of a dosage-dependent regulator of viability can affect the triploid block. Such differential contributions could derive from gene expression in the maternal sporophyte, gametophytes (embryo sac or pollen), or endosperm, or from parentally skewed expression in embryo tissue. There is, as of yet, no practical verification of these possibilities as contributors to postzygotic isolation. Nevertheless, genes exhibit parent-dependent effects on seed development by mechanisms other than imprinting. For instance, altering the development of the female gametophyte can result in growth and differentiation of the seed coat in the absence of fertilization [59–65]. Genes expressed in the seed coat, such as the TRANSPARENT TESTA GLABRA2 (TTG2) transcription factor, control seed development and coordinate the growth of the offspring and the integuments, a maternal organ in the Arabidopsis seed [66]. Ultimately, the molecular identification of genes that affect the triploid block will ascertain whether multiple seed compartments and generations can affect interploidy seed lethality.

Results

Arabidopsis Ecotypes Vary in Paternal Excess Tolerance

To study the mechanism of interploidy seed failure, we investigated the effect of crosses generating paternal excess in different ecotypes of the model plant A. thaliana. Crosses between diploid (2x) seed parents and tetraploid (4x) Ler pollen parents within the Ler or C24 ecotypes produced mainly plump seed (Figure 1 and [42]). In contrast, crosses between 2x Col, Ler, or C24 seed parents and 4x Col pollen parents produced significantly more shriveled seed (Figure 1A). In a previous study, microscopic observation of developing seeds in crosses between 2x and 4x C24 plants showed that the paternal excess phenotype is characterized by overgrowth of endosperm accompanied by delay of endosperm cellularization [42]. In the same study, crosses between 2x C24 seed parents and 6x Col pollen parents produced more extreme phenotypes, including a failure of cellularization, arrest of embryos at the globular to heart stage, and seed abortion. We compared developing seeds from 2x × 4x crosses in the Col genetic background to seeds from 2x × 2x and 2x × 4x crosses in C24. Mature seeds from these three crosses along with confocal images of developing seeds are shown in Figure 1B. At 7 days after pollination (DAP), seeds from 2x × 2x crosses contained embryos at the bent cotyledon stage, and the endosperm was completely cellularized. The 2x
3x4x seeds in C24 at 7 DAP had embryos at torpedo stage, cellularized peripheral endosperm, and uncellularizedchalazal endosperm, showing a delay of embryo development and endosperm cellularization. Seeds from 2x3x4x crosses in Col exhibited an even more severe delay. Col 2x3x4x seeds did not have cellularized peripheral endosperm, and embryos had not passed heart stage even at 8 DAP. Therefore 2x3x4x crosses in Col displayed more features of the previously characterized lethal paternal excess phenotype than 2x3x4x crosses in C24.

The frequency of seed failure was influenced both by the seed parent and the pollen parent. For each of the three ecotypes used as seed parents, crosses were made to Ler and Col tetraploids. Crosses to 4x Col produced more failed seeds, regardless of the maternal genotype (Figure 1A). The highest frequency of F1 lethality (~80% failure, Figure 1) was observed in 2x Col crosses within the Col ecotype. This ruled out divergence-derived genotypic incompatibility, such as hybrid incompatibility [67], as the cause of interploidy seed failure in crosses to 4x Col pollen-parents. Furthermore, the greater survival of seeds derived from 2x Ler relative to 2x Col in crosses to 4x Col pollen parents indicated that a genetic analysis of the maternal control of interploidy incompatibility was possible.

Identification of QTL Responsible for Overcoming Paternal Excess

To map the loci responsible for the differences between the two ecotypes at higher resolution, recombinant inbred lines (RILs) derived from Ler and Col [68] were crossed to 4x Col pollen parents. The rates of seed failure varied among the RIL and differed substantially from the Ler and Col parents (Figure S1). QTL detection and localization by composite interval mapping [69] identified a major locus affecting seed survival in 2x 3x4x crosses on chromosome 2 (Figure 2; permutation adjusted $p = 0.001$). We called this QTL Doctor Strangelove1 (DSL1; [70]). The chromosomal position of DSL1 was estimated to lie between markers gpa1 and bio2b by bootstrap resampling of the data at a 0.90 confidence interval (a table containing the RIL accessions, marker genotypes, and phenotypes is available in Table S1). Ler alleles at this locus were estimated to improve the chance of seed survival in homozygous RIL by 17%, and accounted for 23% of the phenotypic variance in seed survival observed in this population.

The large effect of DSL1 was confirmed using crosses with
chromosome substitution strains (CSS), in which single Ler chromosomes were introgressed into the Col background [71]. CSS lines were crossed to 4x Col, and the percentage of plump to shriveled progeny were compared to those from 2x × 4x Col crosses (Figure S2). The line CSS2, which is homozygous for Ler chromosome 2, including the DSL1 locus, displayed a 38% increase in crossing success relative to the 2x × 4x Col controls. No other Ler chromosome substitutions significantly improved crossing success. Chromosome 2 was further investigated using stepped aligned inbred recombinant strains (STAIRS), which are homozygous for a single chromosomal region of Ler in an otherwise Col background [71]. Seeds from crosses between STAIRS carrying different segments of Ler chromosome 2 and 4x Col pollen parents were assayed for viability using germination tests. Germination frequencies of seeds from the STAIRS used as seed parents and from Col controls were determined (Table S2). Using this method, upper and lower boundaries for the position of DSL1 were established at nga1126 and nga168, which overlaps with the confidence interval for the QTL determined using the RIL population. Thus, we were able to confirm both the large effect and the position of DSL1.

To further refine the estimate of the DSL1 position and investigate the mode of inheritance for DSL1, the frequency of shriveled seed in the progeny of backcross (BC) generations was determined in crosses to 4x Col. Two BC1 (F1 × parental line) populations were generated by backcrossing a Col × Ler F1 to both Ler and Col diploids. The frequency of plump seed was compared to the genotypes of markers located in the DSL1 interval. In both BC1 populations, crossing success increased with increasing dosage of DSL1 alleles (Figure 3A and 3B). The genotype at DSL1 accounted for 16% and 30% of the phenotypic variation in seed set observed in the F1 × Col and F1 × Ler BC populations, respectively. The change from a heterozygous genotype at DSL1 to homozygous DSL1 increases surviving interploidy seeds by 19.2% in the Ler BC1, whereas the change from heterozygous DSL1 to homozygous DSL1 decreased seed survival by 15.0% in the Col BC1 population. These estimates of the magnitude of DSL1’s effect on seed survival are similar to those made in both the RIL and chromosome substitution populations, confirming both the position and large effect of DSL1. In addition, the comparisons of the heterozygote to either homozygote demonstrate that the allelic dosage at the DSL1 QTL additively affects viability.

Individual BC1 (F1 × Col) plants with recombination breakpoints within the DSL1 interval were backcrossed to 2x Col to generate BC2 families. BC2 individuals were genotyped at four markers within the DSL1 QTL: nga361, FIS2, Ve017, and nga168. Progeny from BC2 individuals crossed to 4x Col pollen parents were phenotyped. Statistical significance of t-test comparisons between Col homozygotes and heterozygotes at the FIS2 marker are presented for each BC2 family in Figure 3C. Inheritance of the Ler or Col alleles of a chromosomal region spanning markers nga361 through Ve017, including the FIS2 locus, was not sufficient to change interploidy crossing success. Longer introgressed segments, and segments segregating for the region containing FIS2 and extending through nga168, however, were sufficient to produce the DSL1 effect. Taken together, the RIL, STAIR, and BC2 mapping data suggest that the DSL1 QTL resides between markers Ve017 and nga168.

None of these genetic analyses could distinguish between QTL caused by gene expression in the female gametophyte and postmeiotic gene expression in the female gametophyte or fertilization products. This was addressed using the progeny of a F1 (Ler × Col) × 4x Col cross. Markers linked to DSL1 should depart from the expected ratio 1:1 of Ler:Col in the progeny of this cross if DSL1 acts via expression in the female gametophyte or fertilization products. By contrast, no distortion of allele frequency is expected if DSL1 acts via sporophytic expression, such as via the seed-parent’s vegetative organs, flowers, or in the seed coat. Progeny from F1 × 4x Col (n = 164) were typed for the same markers used in Figure S3 that span the DSL1 interval. Chi-square tests did not detect a significant departure from the 1:1 null hypothesis for any of the markers. The lack of distortion at markers linked to DSL1 argues against rescue of lethality due to postmeiotic expression. The presence of genetic variation sufficient to rescue seed lethality when contributed maternally, but not associated with transmission ratio distortion at linked markers, indicates that the genotype of the maternal sporophyte is critical for the interploidy hybridization barrier.

**DSL1 Participates in an Epistatic Network**

Genome-wide scans for epistatic interactions with DSL1 were carried out by regression analysis. Significance thresholds for the detection of epistasis were set by permutation and estimation of the effect of all marker pairs plus their interaction in each permuted set. Regressions using two markers and their interaction with combined effects that exceeded this threshold were identified (for a complete table,
The standard deviations) in BC1 families from F1
Figure 3. Fine Mapping of (A and B) The mean frequencies of plump seed (y-axis, error bars indicate the standard deviations) in BC1 families from F1 × Ler (A) and F1 × Col (B) BC1 families genotyped at the FIS2 marker (x-axis) and crossed to 4x Col. Heterozygotes and homozygotes were different as determined by t-test at p < 0.05 in both populations. (C) Illustrations of the genotype of BC2 families (grey, white, and black boxes) segregating for chromosomes recombinant within the DSL1 interval. The p-values to the right of the illustrations indicate whether segregation of that interval resulted in segregation of the DSL1 QTL as determined by t-tests comparing the mean proportion of plump seed in progeny of crosses between 4x Col and BC2 seed-parents heterozygous or homozygous for segregating molecular markers.
doi:10.1371/journal.pbio.0060308.g003

see Table S3). These putative epistatic interactions were reexamined by multiple regression to ensure that the interaction component significantly improved the fit of the model to the data.

Two loci, EPISTASIS OVERCOMES PATERNAL EXCESS1 (EOP1) and EOP2, comprising multiple markers on chromosome 3 and chromosome 5, respectively, affected seed survival when considered in conjunction with the genotype at DSL1 (Figure 4 and Table S3). The effect of the DSL1-linked marker g17288 on the percentage of surviving seeds in RIL × 4x Col crosses displayed a significant interaction with the EOP1 and EOP2 linked markers nga162 and nga139, respectively. Regression models of the DSL1-EOP1 and DSL1-EOP2 interactions were significant at a permutation-test-adjusted p < 0.01, and the sum of the interaction terms accounted for as much as 15% of the phenotypic variation in the RIL population. The Ler alleles of either EOP1 or EOP2 increased the proportion of plump seed. In DSL1Col plants, EOP1Ler increased the proportion of plump seed in crosses to 4x Col (Figure 4A), whereas in the DSL1Ler background, the EOP1Col allele caused a slight, but insignificant, increase in crossing success. The effect of the other epistatic locus, EOP2, on the frequency of seed abortion is only observed in the DSL1Col background. The combination EOP2Col and DSL1Col resulted in a higher frequency of plump seed formation. Estimated jointly, EOP1, EOP2, and DSL1 plus their interactions accounted for over 40% of the phenotypic variance in seed viability in Ler × Col RILs crossed to 4x Col (Table S4).

The effect of EOP1 on cross fertility was strong in the RIL, so the mode of EOP1 action was investigated by transmission ratio test. If EOP1 acts via the genotype of the fertilization products or embryo sac, it should exhibit a distorted ratio in interploidy crosses between Col × Ler F1 hybrids and 4x Col. If, like DSL1, the interaction occurs between alleles in the maternal sporophyte, no distortion should be observed. Markers linked to EOP1, nga172, and nga162 were genotyped in the F1 × 4x Col progeny. Consistent with a postsegregation requirement for EOP1 in interploidy seeds, the genotype at nga172 was distorted in triploid (X2 p-value = 0.0008, observed heterozygotes [Het] = 109, Col = 65 vs. expected Het = 87, Col = 87), but not in diploid progeny (p-value > 0.05; Het = 47, Col = 54). A similar trend was observed at nga162, but it was not significant. The detection of interploidy-dependent transmission ratio distortion confirms the presence of a QTL on chromosome 3 that affects interploidy viability via expression in the female gametophyte, endosperm or embryo.

TTG2 Regulates the Interploidy Barrier

We sought to determine which gene within the DSL1 interval could be responsible for the phenotypes observed. The imprinted gene FERTILIZATION INDEPENDENT SEED2 (FIS2) is near the DSL1 interval, and the Col allele has a 180-bp in-frame deletion in the coding region with respect to Ler and C24 [65]. Another candidate gene in the DSL1 interval is TTG2. TTG2 encodes a WRKY transcription factor that controls epidermal cell fate with pleiotropic effects on seed development and trichome production [72]. Seeds from mothers homozygous for tgg2 loss-of-function mutations have a pale seed coat because the innermost layer of the seed coat (the endothelium) fails to accumulate pigments; in addition, the epidermal layer of the outer integument does not
we tested branch number \[72,73\]. have reduced leaf trichome density and decreased trichome precocious endosperm cellularization [66]. The mutants also exhibiting reduced cell elongation in integuments, and FIS2 interploidy lethality. Homozygous loss-of-function mutants at FIS2 produce mucilage [72]. ttg2–1 mutants produce small seeds in crosses to 4x Col to 64% (\( n = 289 \)) plump seed were observed in crosses to 4x Col pollen parents, over 78% of seeds were plump (\( n = 136 \)). This was significantly different from wild-type Ler seed parents, which produced 52% (\( n = 198 \)) plump seed in crosses to 4x Col. Similarly, the percentage of plump seed increased from approximately 14% (\( n = 111 \)) to 64% (\( n = 111 \)) when ttg2–3 mutants were substituted as seed parents. TTG2ttg2–3 heterozygotes exhibited a slight increase in fertility, but the trend was not significant (Figure 5B, \( t\)-test \( p > 0.05 \)). Thus, loss-of-function alleles at TTG2 reduced the interploidy hybridization barrier.

Consistent with TTG2 encoding the DSL1 QTL and the Ler genome encoding a weak allele of TTG2, the degree of rescue provided by a loss-of-function allele in Col was many-fold greater than in Ler.

No polymorphisms between the Ler and Col coding sequence of TTG2 were found. We therefore sought to determine whether an expression level polymorphism differentiates the alleles. Single nucleotide polymorphisms (SNPs) present in the TTG2 transcript were identified in the data from chip-based resequencing of \( A.\) thaliana [78]. A SNP present in the 5’ UTR of the full gene (PERL030427) was confirmed by sequencing of PCR reactions using genomic DNA from the Ler and Col ecotypes as template. The proportion of each allele was measured in the amplified...
products of DNA and cDNA prepared from Ler × Col F1 hybrids. By measuring the proportion of the two alleles that accumulated in an F1 hybrid, the two alleles were tested in an identical regulatory environment at equal gene dosage, and any differential allelic contribution must result from a cis-regulatory difference between the two alleles. The proportion of TTG2 mRNA contributed by the Ler allele to the reverse transcription PCR products was approximately 20% less than genomic DNA samples (t-test p-value, 0.001) (Figure 5C). This indicated that the Ler allele accumulates less TTG2 message, consistent with the Ler encoding a weak TTG2 allele and the relative effects of the ttg2–1 and ttg2–3 knockout alleles in improving interploidy cross fertility.

The hybridization-based resequencing data did not clearly indicate any polymorphisms that might explain the difference in expression. To determine what, if any, putative cis-regulatory polymorphisms exist, the DNA sequence immediately upstream of the presumptive transcriptional start site was determined in Ler. At the −53 position, relative to the start of the longest TTG2 cDNA, and 11 bp downstream of the previously identified Werewolf, Glabrous1, and Transparent Testa2 transcription factor binding sites [73], Ler contains a 7-bp insertion of the sequence AGACCAA between the nucleotides ACGTTCAACGAGTGTCCAT and a 26-bp stretch of pyrimidines, beginning with CTCCCTC. This insertion is 43 bp 5' of the presumptive TATA box of the TTG2 promoter. Consistent with the presence of an insertion, the hybridization-based resequencing data [78] show hybridization failure in microarrays hybridized with Ler, but not Col, labeled genomic DNA beginning with probes 8 bp upstream of the insertion site (http://signal.salk.edu/perlegen.html). Amplification of genomic DNA from Col and Ler using PCR primers flanking the insertion confirmed a fragment-
length polymorphism distinguishing Col and Ler that could be resolved on an agarose gel (unpublished data).

Cellularization of the Peripheral Endosperm Is Associated with Rescue of Interploidy Seeds

We next examined developing seeds from crosses between Col, Ler, ttg2–3, ttg2–1, or CSS2 seed parents and 4x Col pollen parents. Phenotypes associated with lethality and rescue were observed by confocal and differential interference microscopy. Confocal images of seeds at 6 DAP are shown in Figure 6. All crosses displayed features we have previously observed in seeds with paternal excess, including enlarged chalazal endosperm and nodules, and delays of embryogenesis and of endosperm cellularization. We examined the size of the chalazal endosperm and nodules, since these were previously observed to be larger in seeds with lethal than with viable paternal excess [42]. Maximum cross-sectional areas of the embryo sac and of chalazal endosperm plus nodules were measured on digital photographs of cleared whole-mount seeds. We found no clear relationship between embryo sac size at 7 DAP (the latest time point when cleared seeds could be imaged) and viability, as ttg2–1 × 4x Col seeds had smaller embryo sacs than 2x Ler × 4x Col, but ttg2–2 × 4x Col embryo sacs were a similar size to those of 2x Col × 4x Col (Figure 7B). This ruled out reduction of integument growth as a general factor in seed rescue. Neither was a restriction in size of the chalazal endosperm and nodules required for seed fertility, as Ler × 4x Col seeds had larger chalazal endosperm and nodules than Col × 4x Col seeds, in both absolute and relative terms, at 7 DAP (Figure 7C and 7D). In fact in ttg2–1 × 4x Col, these structures occupied nearly twice the relative area as in Col × 4x Col. The relative area of the seed cavity occupied by chalazal endosperm and nodules was greater in Ler and ttg2–1 than in Col, ttg2–3, and CSS2, demonstrating additional variation in endosperm developmental programs in Ler and Col.

The frequency and timing of endosperm cellularization was determined by examining confocal micrographs of developing seeds. The frequency of endosperm cellularization was consistently increased in all comparisons that improved interploidy crossing success (Figure 7A). By 8 DAP, nearly all ttg2-1 × 4x Col seeds contained cellularizing endosperm, whereas only some Ler × 4x Col showed signs of endosperm cellularization. Similarly, seeds from ttg2–3 × 4x Col were intermediate in the frequency of cellularization, but hardly any 2x Col × 4x Col seeds showed signs of cellularization. CSS2 × 4x Col likewise was more frequently cellularized at 8 DAP than 2xCol × 4x Col. Similar results were obtained from observations at 7 DAP and 6 DAP (unpublished data). We concluded that TTG2 action in the sporophyte delays endosperm cellularization in paternal excess crosses, and this hinders embryo development.

Discussion

Postzygotic hybridization barriers restrict gene flow and isolate populations from one another. It has long been known that shifts in ploidy can affect postzygotic lethality and create isolated populations of different ploidy. This kind of F1 lethality can occur in the absence of any allelic diversity. The presence of such barriers has been proposed as one of the mechanisms by which shifts in ploidy can potentiate the process of speciation [9,21,79]. Yet, considerable variability in this trait exists within species and between species [9,41]. We investigated the molecular mechanism responsible for variation in the lethality of interploidy crosses in Arabidopsis.

The genotype of both the paternal and maternal lineages caused variation in the postzygotic lethality of 2x × 4x crosses (Figure 1). In agreement with previous studies [42,55], interploidy crosses using accessions Ler and C24 produced a majority of live seed whether within or between genotypes. Crosses using diploid Col seed parents and either Ler or C24 tetraploid pollen parents also resulted in a majority of well-formed plump seeds. When Col tetraploids were used as
pollen donors, however, a dramatic increase in the frequency of failed seeds occurred in crosses to all three maternal backgrounds. Importantly, the loss of viability was not due to genetic incompatibilities between accessions, as the least compatible cross was 2x Col × 4x Col in which identical genotypes were contributed by both parents at different dosages. The expression of variation via either parent indicates that the genetic basis must either be dosage sensitive, i.e., additive, or result from independent maternal and paternal effects. Like earlier studies [42,55], the initial crossing design could not distinguish between additivity, parental effects mediated by parent-dependent genomic imprinting, or maternal sporophytic, cytoplasmic, or gametophytic effects. Any or all of these could contribute to interploidy lethality [51].

**Dr. Strangelove1 and an Epistatic Network Control**

**Interploidy Lethality**

A single QTL, called *Dr. Strangelove1*, was detected using the fertility of Ler × Col RIL crossed to 4x Col as a quantitative trait (Figure 2). The effect of the genotype at *DSL1* was also observed when BCs of the F1 to both Ler and Col were crossed to 4x Col, indicating that the phenotype was sensitive to the dosage of *DSL1* alleles in the maternal parent. The maternal contribution of Ler alleles was sufficient to rescue part of the lethality, and alleles were not subject to transmission ratio distortion in F1 × 4x Col crosses. Thus, the genotype of the maternal sporophyte was likely responsible for the action of the QTL. This validates the proposition that gene regulation during all life cycle stages must be considered as possible explanations for variation in seed fitness, parent-of-origin effects, and hybridization barriers, and not only genomic imprinting in the zygotes [51]. Indeed, a requirement for the coordination of the maternal sporophyte and endosperm development suggests that parent–offspring conflict could cause the observed variation.

Two additional QTL, *EOP1* and *EOP2* (Figure 4), interact with the genotype of markers linked to *DSL1*. This limited genetic network modulated seed lethality and contributed approximately 20% of the phenotypic variance in seed survival in the RIL population. A similar, but more extensive, network of epistatic loci has been found to affect variation in *A. thaliana* accessions F1 hybrid fitness in crosses to *A. arenosa* (C. Josefsson, B. Dilkes, and L. Comai, unpublished data). Such “intragenomic” epistasis, distinct from epistasis expressed due to the divergence between species, may be a general feature of hybridization barriers that arises due to the dosage-sensitivity of the genes responsible for F1 lethality. Dosage-sensitive genes are more likely to encode members of signaling cascades and protein interaction networks [80]. Such networked gene products would be more susceptible to interlocus variation and epistasis. Moreover, selection on a trait affected by a network could result in the accumulation of mutations at multiple interacting genes.

We made no estimates of the confidence intervals for the positions of *EOP1* or *EOP2*. Nevertheless, the finding that...
TTG2 (discussed below) could affect the interploidy barrier suggests that loci with demonstrated genetic interactions with TTG2 such as the leucine-rich receptor-like kinase Haiku2 (IKU2) [66] may also possess the ability to regulate the ploidy barrier. Similarly, upstream regulators of TTG2, such as the transcription factors TTG1 and TT2, which are required for TTG2 expression [81], might also modulate interploidy lethality. Interestingly, IKU2 and TTG1 map to the same chromosome arms as EOP1 and EOP2, respectively.

TTG2 and the Control of Interploidy Lethality

The transcription factor TTG2, which controls seed development via expression in the maternal sporophyte, was located within the chromosomal interval containing DSL1 [66,72]. Seeds from ttg2–1 mutant sporophytes possess defects in both sporophytic (seed coat differentiation) and zygotic (precocious endosperm cellularization) traits. We propose that allelic variation at TTG2 could explain the DSL1 QTL. Loss-of-function mutations in TTG2 isolated from both the Ler and Col backgrounds improved interploidy crossing success (Figure 5B). DSL1 is an additive QTL, yet ttg2–I has been shown to be recessive for most seed size traits [66] and is reportedly recessive for trichome density [72]. Nevertheless, at least one comparison between ttg2–II+ and ++ maternal crosses, seed width, was reported as significantly different (see Table 1 in [66]), demonstrating that a single wild-type TTG2 locus can be insufficient to support completely normal seed growth in balanced crosses as well.

Additional phenotypes are consistent with Ler encoding a weak TTG2 allele. A QTL affecting reduced trichome density and linked to TTG2 has been described previously [75–77]. The effect of this QTL on trichome density is additive [77], just like DSL1 (Figure 3). Seed color in Ler is also lighter than Col, as would be predicted [82]. These data are all consistent with the Ler allele at DSL1 encoding a hypomorphic allele of TTG2. The alleles have not been transgenically tested for differential effects on the hybridization barrier, but a cis-regulatory difference in expression of the alleles was detected in Ler/Col heterozygotes. The difference in transcript level for this transcription factor is consistent with the type of variation that can underlie QTL function. Regardless, the large single-gene effect in ttg2–I and ttg2–3 mutants observed here demonstrates that reproductive isolation by ploidy and fertility restoration can be mediated by single mutations expressed in the sporophyte.

Control of Cellular Differentiation in the Endosperm and the Mechanism of DSL1- and TTG2-Mediated Fertility

The earliest evidence of aberrant development in the progeny of paternal excess crosses was visible in the endosperm [42]. Endosperm failed to progress from the free nuclear proliferation typical of early development to cellularized mitotic proliferation, which normally occurs at the globular to heart transition of embryogenesis. The lack of endosperm cellularization in 2x Col × 4x Col crosses is similar to that observed in A. thaliana × A. arenosa crosses [49] and the heterochronic shift described in fertilized seeds of mutants affected in the Arabidopsis orthologs of Polycomb-Repressive Complex 2 subunits [59–65,83]. Paternal excess crosses of diploid Ler or Col and tetraploid Col differed in their respective endosperm growth phenotypes. Cellularized peripheral endosperm was observed more frequently in crosses to Ler seed parents than in Col seed parents (Figure 7A). Rescue of interploidy lethality by substituting the chromosome containing DSL1 from Ler into a Col background was also associated with more frequent observation of cellularized peripheral endosperm in developing seeds.

TTG2 was identified as an effector of plant development. The ttg2–1 allele in the Ler background has a maternal effect on seed size and induces early cellularization of the endosperm in balanced crosses [66,84]. Loss of TTG2 from diploid maternal sporophytes can result in seed failure due to an interaction with the haiku2–1 (iku2–1) mutation [66], which also decreased seed size and induced early cellularization of the endosperm. This demonstrated that proper TTG2 function can be required for viability. Similar to the effects in balanced crosses, ttg2–I × 4x Col crosses also produced seeds with precocious endosperm cellularization as compared to wild-type Ler × 4x Col (Figure 7). This rescue of interploidy fertilization by maternal transmission of TTG2 loss of function alleles, demonstrated here, further supports the placement of this gene in a pathway coordinating the growth of the maternal tissues and fertilization products.

One proposed mechanism for the effect of TTG2 on endosperm cellularization in balanced crosses is that the reduced growth of maternal tissue in ttg2 mutants restricts embryo sac expansion and promotes endosperm cellularization [85]. Mature seeds produced by a self-pollinated ttg2–I mutant are notably smaller than wild type, and ttg2–3 seeds are slightly smaller. Yet, embryo sacs at the time of endosperm cellularization from ttg2–3 × 4x Col crosses were no smaller than those of the uncellularized proliferating Col × 4x Col. Thus, loss of TTG2 function in Col appears to promote endosperm cellularization independent of seed or ovule growth. Alternative explanations include a change in signaling molecule release or perception due to loss of TTG2. Proanthocyanidins, which were markedly reduced in ttg2–I seeds [66,72], decrease the permeability of the endothelium, the maternal cell layer adjoining the endosperm [82]. Their absence in the endothelium of ttg2 mutants may allow diffusion of a molecule that promotes endosperm differentiation. Alternatively, ttg2 mutations may prevent normal differentiation of the endothelium [82], and thereby disrupt the production of a signal from the seed coat to the endosperm that delays cellularization in wild-type plants.

Evolutionary Consequences of Variation in Interploidy Barriers

A stronger postzygotic hybridization barrier, such as provided by the Col genome, would restrict allele sharing between diploids and tetraploids. In the absence of gene flow, independent adaptation of the two populations and/or
speciation would be possible. Even complete interploidy block might not result in polyploid speciation as new alleles arising in diploids can be shared with tetraploid populations via unreduced gametes or recurrent polyploid formation. The relative absence of a crossing barrier, however, would result in more frequent triploid production. Triploids of *Arabidopsis* produce aneuploid swarms that resolve into diploid and tetraploid cytotypes [15,44]. More frequent swarm production would increase the opportunities for karyotype evolution, selection of alleles in novel and unbalanced karyotypes, and both diploid and tetraploid descendants from a triploid would facilitate gene flow between diploid and tetraploid cytotypes. This is expected to strongly inhibit the establishment of new polyploid species, but to increase the chance of finding polyploid cytotypes within a diploid population [1,2,5,9]. Thus far, surveys of the nuclear DNA content from a few hundred *Arabidopsis* accessions have identified only two tetraploids [44,86]. A study utilizing crosses between 288 accessions of *Arabidopsis* identified one additional tetraploid accession on the basis of increased seed lethality in the intercross progeny [67]. Rates of 2n gamete formation in pollen meioses of *A. thaliana* have been estimated to be an order of magnitude lower than these observed frequencies of tetraploid accessions, at approximately 1/3,000 [87]. Given these low frequencies and the low rate of outcrossing in *A. thaliana* [88,89], selection on mutations that prevent triploid offspring from unreduced gametes or sympatric tetraploids seems unlikely. Within-species variation for the ploidy hybridization barrier is not unprecedented (e.g., [41]) and may result from selection on seed performance. The genes that have been hypothesized by some to underlie the interploidy barrier might be under continued selection due to the ability of variation in expression to affect seed fitness [51] or purifying selection to maintain optimal expression regulation [90,91]. Despite these suggestions, it is possible that neutral variation, i.e., of no consequence in balanced crosses, limits fitness in the out-of-balance fertilization products produced by interploidy crosses. Further study of the genes and alleles identified here will be necessary to determine whether they are subject to selection in diploid populations. The identification of *TTG2*, which also determines trichome density, as a determinant of variation in interploidy postzygotic lethality suggests that pleiotropy may confound the interpretation of such results. For instance, in the absence of herbivore pressure, trichome density is negatively associated with *Arabidopsis* fitness, whereas it is beneficial in the presence of herbivores [92]. *TTG2* alleles selected for their effect on trichome density, but neutral with respect to seed fertility in balanced crosses, may result in differences in seed lethality following interploidy hybridization.

**Epigenetics and Development Converge in Plant Hybridization**

Previously, it has been shown that disruption of gene silencing via loss of CG methylation can disrupt both interploidy survival [55] and interspecies hybrid survival [49]. The disruption of imprinting in the species hybrids may destroy the coordination of the two maternal programs (those expressed in the seed and those in the integuments), resulting in seed failure. In this sense, intergenerational control of seed development is responsible for maternal control of both the species barrier and ploidy barrier (this study; [48,49]). Loss of imprinting in interspecies hybrids was associated with epigenetic disturbance affecting both heterochromatin and genes [48], and chromatin and DNA methylation disturbances have been noted in interploidy crosses of both *Arabidopsis* and maize [93,94]. In this study, we demonstrate that a compensatory change in the maternal genotype can overcome these epigenetic disturbances. Although it would appear that there are two, as yet, nonoverlapping mechanisms affecting the strength of the interploidy hybridization barrier, they may have more in common. It is reasonable to propose that the mechanism identified here might interact with targets of CG-dinucleotide methylation that coordinate the growth and development of seeds. For example, it is possible that runaway proliferation in the endosperm caused by misregulation of MEDEA, an imprinted gene and known target of CG methylation, could be opposed by reduced *TTG2* function.

**Materials and Methods**

**Plant materials and growth.** Tetraploids of *Arabidopsis* ecotypes Col-0 and Ler were produced by submerging seedlings for 2 h in an aqueous solution of 0.1% colchicine (Sigma). Seedlings were subsequently washed in copious quantities of water, and transplanted to soil. Treated plants were grown to maturity and the basal nuclear ploidy of the progeny determined by flow cytometric analyses of nuclear DNA content following isolation of nuclei from leaves or inflorescence buds as previously described [44].

**RILs** derived from a cross between Col and Ler [68] were provided by the *Arabidopsis* Biological Resource Center (ABRC). STAIRS were provided by Michael Kearsey (University of Birmingham, United Kingdom). A male-sterile C24 A9 barnase line described by Paul et al. [95] was crossed to Col-0 for ten successive generations to produce “Col A9” (gift of Rinke Vinkenoog). Col-0/Ler F1 hybrid individuals were crossed to Col A9 male-sterile plants or to hand-emasculated Ler plants to generate reciprocal BC1 families. Col A9 male-sterile plants were crossed to Ler to produce male-sterile F1 plants used to test transmission ratio distortion in crosses to 4x Col. *ttg2–2* was a gift from David Smyth [72]. The novel allele *ttg2–3* was derived from the SALK insertion line 149938 [96]. The *fs2–8* allele [97,98] was provided by Ramin Yadegari and Kristen Newcomb (University of Arizona, Tucson, Arizona), who backcrossed the mutation in the Col background for seven consecutive generations. The Col × Ler RIL population was grown in Washington greenhouse with ambient light supplemented to make a 16-h light:8-h dark day. Temperatures were set for 18°C during the day to 21°C during the night, but varied with atmospheric conditions. All other experiments were carried out in controlled chamber conditions under 16:8-h light:dark and 21:18°C day:night temperatures. Crosses for the RIL, BC1, and TRD experiments were performed by emasculating inflorescences by removing all organs from the outer three whorls with jewelers forceps. Crosses to the STAIRS were done by removing only the stamens and pollinating 2 d after emasculation. STAIRS and control crosses were grown at the University of Bath in a Sanyo controlled environment room with a day length of 16 h, at 29/18°C day/night temperatures. For comparison of *ttg2* mutants and wild type, seeds were weighed with a Mettler UMT2 microbalance (Mettler-Toledo).

**Genetic analyses.** For the analysis of RILs, marker genotypes were obtained from the European Union (EU) Natural program (see also, http://arabidopsis.info/new_ri_map.html). Only genetic markers with data for 95% or more of the lines were used for mapping. Markers with no recombination between them were removed from the analysis using the MapManagerQTX 2.0 package [99]. Markers and genotypes for the RIL used in the analysis can be found as Table S4. Three individuals from each RIL were crossed to 4x Col, and the numbers of plump and shriveled seeds were counted. The frequency of plump seeds for each individual was defined as the trait, and RIL means were computed from at least three replicates. QTL identification and likelihood estimation were performed using the mean RIL values under models 1 and 6 in the QTL Cartographer software package [100]. Statistical thresholds for QTL identification were set by 1,000 permutations according to the method of Churchill and
Doerge [101]. Estimates of QTL position in the RIL were made by 1,000 bootstraps with replacement followed by interval mapping [102]. The effects of genomic loci segregating between STAIRS and controls or among BC1 populations on the frequency of plump seed following crosses of Col-0 with Col-0 were tested by t-test and PCR. Transmission ratio distortion was evaluated by chi-square comparisons of observed allele frequencies to expected (1:1) frequencies in the progeny of crosses of F1 or BC1 individuals heterozygous for informative markers to a 4x Col-0 pollen parent. All analyses for multivariate analyses and epistasis confirmation were performed using the JMP statistics program (Ver. 5.1, SAS institute).

Epistatic QTL were identified using MapManagerQTX by regression on every possible marker pair. Statistical thresholds for epistasis detection were set by 1,000 permutations of the data followed by regression on every possible marker pair. Epistatic interactions were considered putative if they exceeded a chi-square value of 28.6, which corresponded to the permutation-adjusted p-value of 0.05. Epistatic interactions were considered confirmed if the interaction term was significant in linear regressions of both marker and the interaction term on the mean RIL data (y = M1 + M2 + M1 × M2; where y corresponds to the mean plump seed frequency and M1 and M2 are marker genotypes).

Marker genotypes of BC1 and BC2 individuals were determined at nga361, nga168, and nga102 by autoradiography of dried gels following electrophoresis of 2P alpha-CTP incorporation-labeled PCR products in 6% polyacrylamide (35:1 acrylamide to bis acrylamide) in 0.5 × Tris borate EDTA by standard protocols [103]. Markers nga172, Ve017, TTG2, and FIS2 were analyzed by phototraphy of ethidium bromide fluorescence from electrophoresis in agarose and 0.5 × TBE by standard methods. PCR products from primers amplifying marker Ve017 was digested as recommended by The Arabidopsis Information Resource (http://www.arabidopsis.org). All primers are given in Table S8.

eQTL determination by SNP quantitation at TTG2. Genomic DNA and total RNA were extracted from diploid Ler, Col-0, and F1 hybrid young leaves. cDNA was generated using the gene-specific primer tgg2_2bottomR (CTTCTCTCCCTGGACTCAGC) and superscriptII Reverse transcriptase according to the manufacturer's instructions (Invitrogen). PCR primers (ttg25 primer: tgg25_snpF: ggcatccatccaacgttcccta; ttg25_snpR: cagcatcaatcagcttcca) spanning the first intron of the TTG2 transcript (NUBIS). We thank the Yadegari, Kearsey, Manly, and Smyth for supporting the Nikon and University of Bath Imaging Suite (BBSRC). We thank the National Science Foundation Plant Genome Research Program, Polyploidy Project (NSF DBI 0077774 and DBI 0501712 to LC), the National Institutes of Health (1R01GM076103 to LC), the U.S. Department of Agriculture (USDA) National Research Initiative (2003-35000-13248 to BPD), and the Biotechnology and Biological Sciences Research Council (BBSRC), UK (grant no. BB/D001234/1 to DL).

Acknowledgments

Supporting Information

Figure S1. Distribution of the Percentage of Live Seed for Each Ler × Col RIL

Table S1. Marker genotypes of Col × Ler RIL used in QTL identification

Table S2. STAIRs Localization of DSL1 Position on Chromosome 2

Table S3. Summary of Significant Pair-Wise Marker Interactions Affecting Interploidy Lethality in Ler × Col RIL Crossed to Tetraploid Col

Table S4. Main and Interaction Effects for % Live Seed in Col × Ler RIL

Table S5. Did Not Modulate Viability in Crosses to Tetraploid Col

Table S6. TTG2 Modulates Mature Seed Size in Ler and Col Genetic Backgrounds

Table S7. TTG2 Modulates Seed Viability in Crosses of Diploid Mutants to Tetraploid Col

Table S8. Oligonucleotide Primers Used in This Study

References


Lethality in Interploidy Crosses


83. Lethality in Interploidy Crosses
