Title

Cleave and Rescue gamete killers create conditions for gene drive in plants

Authors

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Abstract

Gene drive elements promote the spread of linked traits, even when their presence confers a fitness cost to carriers, and can be used to change the composition or fate of wild populations. Cleave and Rescue (*ClvR*) drive elements sit at a fixed chromosomal position and include a DNA sequence-modifying enzyme such as Cas9/gRNAs (the Cleaver/Toxin) that disrupts endogenous versions of an essential gene, and a recoded version of the essential gene resistant to cleavage (the Rescue/Antidote). *ClvR* spreads by creating conditions in which those lacking *ClvR* die because they lack functional versions of the essential gene. We demonstrate the essential features of *ClvR* gene drive in the plant *Arabidopsis thaliana* through killing of gametes that fail to inherit a *ClvR* that targets the essential gene YKT61, whose expression is required in male and female gametes for their survival. Resistant (uncleavable but functional) alleles, which can slow or prevent drive, were not observed. Modeling shows plant *ClvR*s can be used to rapidly drive population modification or suppression. Possible applications in weed control, plant breeding and conservation are discussed.

Keywords

Gene drive, *Arabidopsis thaliana*, selfish genetic element, population modification, population suppression, weed, crop hybridization, conservation, palmer amaranth

Main

Gene drive occurs when genetic elements—genes, gene complexes, large chromosomal regions or entire chromosomes—are transmitted to viable, fertile progeny at rates greater than those of competing allelic variants or other parts of the genome. There has long been interest in the idea that genetic manipulation of wild populations via gene drive could be used for beneficial purposes. Transgenes or alleles of endogenous loci can be linked with a drive element. The results of modeling and lab experiments demonstrate this can result in spread of these “cargo” genes to high frequency in an extant population. Alternatively, drive can result in population suppression or elimination if spread of the element drives the population towards an unfit set of genotypes (e.g., all male, all females sterile), reviewed in[1–3].

A variety of selfish genetic elements have been considered for bringing about gene drive. These include transposons and homing endonucleases, which spread through over-replication (copying); multigene complexes that bring about female meiotic drive or sperm post-meiotic segregation distortion; and toxin-antidote combinations that cause the death of those (cells, spores, gametes, or progeny) who fail to inherit them from a carrier. Toxin-antidote gene drive elements (TA elements) are particularly interesting as they are found throughout all domains of life: prokaryotes, fungi, animals and plants; and the wide distribution of some of these elements in nature shows they can spread

and persist in complex natural environments[4–6]. Here we focus on eukaryotes and drive associated with sexual reproduction.

A TA element sits at a fixed chromosomal position and consists of one or more genes that encode linked toxin and antidote functions. The toxin, typically a protein, has the potential to kill or impair the development of those in which it is present, while the antidote, a protein or RNA, suppresses the toxin's activity or expression[4,7]. The toxin is trans-acting and is distributed to all meiotic products or progeny of a TA-bearing parent. However, only those that inherit the TA cassette express the antidote, which counteracts the toxin in cis. In consequence, TA elements ensure their presence in the next generation by causing the death of those who fail to inherit them (post-segregational killing), a form of genetic addiction. The death of those lacking the TA cassette can result in a relative increase in frequency of those carrying it. Modeling shows that TA elements in sexually reproducing eukaryotes can (depending on the fitness costs associated with carriage of the element and introduction frequency) spread to high frequency even if they do not confer any advantage to their hosts[8–18].

TA elements that exist in nature evolved in specific genomic, organismal and ecological contexts, and it is often unclear if the mechanisms of action, associated gene regulation and species-specific information on development (timing and levels of gene and protein expression and localization) can be easily transferred to bring about drive in other

species. Similar considerations apply to synthetic *Medea* TA elements engineered in *Drosophila* in which the toxin is an engineered transient loss-of-function LOF) of a maternally expressed gene whose product is essential for embryogenesis and the antidote is a zygotically expressed transgene that restores this missing function in a just-in-time fashion[19–21].

Recently, in an effort to create a chromosomal TA-based gene drive system that utilizes a LOF toxin and consists of a simple and extensible set of components that can plausibly be implemented across diverse species we developed the *Cleave and Rescue* (*ClvR*) element[14,22–24], also referred to as Toxin Antidote Recessive Embryo (TARE)[15,25] in related implementations (hereafter referred to as *ClvR*, a name that captures the key mechanisms involved). A *ClvR* element encodes two activities. The first component, the Cleaver/Toxin, is a DNA sequence–modifying enzyme such as Cas9 and multiple guide RNAs (gRNAs). These are expressed in the germline or cells that will become the germline, though germline-specific expression is not required. In the case of Cas9, it disrupts—through cycles of cleavage and end joining that continue until the target site is destroyed—endogenous versions of a haplosufficient (and in some contexts haploinsufficient or haplolethal) essential gene, wherever it is located. Inaccurate repair at multiple positions in the coding region of the essential gene creates loss-of-function (LOF) alleles. These are the potential toxin. The second component of *ClvR*, the Rescue/Antidote, is simply a recoded version of the essential gene resistant to cleavage and gene conversion with the cleaved version, expressed under the control of

regulatory sequences sufficient to rescue the LOF phenotype. LOF alleles of the essential gene, which segregate and exist independently of *ClvR*, perform their toxin function when they find themselves (potentially many generations later) in homozygotes that die because they lack the *ClvR*-derived source of essential gene function. In contrast, those who inherit *ClvR* and its associated Rescue survive. In this way, as with TA-based selfish genetic elements found in nature, *ClvR* spreads by causing the death of those that lack it. This results in cells, organisms, and ultimately populations becoming dependent on (addicted to) the *ClvR*-encoded Rescue transgene for their survival.

In *Drosophila*, autonomous *ClvR*/TARE elements have been created and shown to spread in wildtype (WT) populations to transgene fixation (all individuals carry at least one copy)[14,15,22,24,25]. Other features, such as the ability to bring about strong, but self-limited drive[23], engage in multiple cycles of population modification that replace old content with new[22], and bring about population suppression using a conditional Rescue[24] have also been demonstrated. Multiple other configurations of the components that make up *ClvR*/TARE have been proposed, and modeling predicts they can bring about drive in animals with a diversity of interesting characteristics for population modification or suppression[16,26].

In animals *ClvR*-type drive is most easily implemented through the killing and rescue of specific zygote genotypes. TA drive based on killing and rescue of gametes is challenging for several reasons. In females the gamete is chosen through differential segregation of one of the products of meiosis within the common cytoplasm of the oocyte and thus there is no opportunity for the antidote to select for carriers. In spermatogenesis the haploid spermatid products of a meiosis are connected by cytoplasmic bridges until late in development, and active content sharing of many but not all products (e.g.[27]) limits opportunities for bringing about differential killing and survival[28].

In our original description of *ClvR* we noted that gametic drive could be implemented in other sexual organisms, such as fungi and plants (Fig. S1 in[14]), in which sibling gametes do not share components and require haploid gene expression for development and/or survival. In plants, meiotic products undergo additional rounds of mitosis, developing into multicellular haploid gametophytes (the female megagametophyte and male microgametophyte) that produce egg or sperm and other essential support cells. This requires extensive expression of the haploid genome[29]. These features of plant gamete development are reflected in the many recessive mutants (no somatic phenotype in the heterozygous diploid or polyploid parent; the sporophyte stage) that cannot be transmitted through one or the other sex, often identified through sex-specific transmission ratio distortion (e.g.[30,31]). These characteristics make plants an ideal

system in which to bring about gene drive based on a *Cleave and Rescue* mechanism that causes the death of gametes that fail to inherit *ClvR* from a parent (Fig. 1a-d).

Arabidopsis thaliana is a good system in which to test self-sustaining gene drive constructs in plants because it is a self-fertilizing hermaphrodite in which fertilization typically occurs before flower opening, thus limiting opportunities for pollen/gene flow. In addition, *A. thaliana* is not naturally wind pollinated and lab and field experiments demonstrate that outcrossing rates are very low[32–35]. Thus, transgene containment is straightforward. However, for these same reasons, population level gene drive experiments of the type carried out in insects—mixed populations of transgenic and non-transgenics allowed to mate freely and followed for changes in genotype frequency over multiple generations—cannot be carried out. Here we show, using manual mating between parents of different genotypes, the key features required for *ClvR* drive: a high frequency of LOF allele creation, a high, non-Mendelian rate of *ClvR* inheritance in progeny, and the absence of resistant alleles (mutated, uncleavable, but functional) that would slow or subvert the intended goal of drive, population modification or suppression. Modeling shows that elements with the features we demonstrate experimentally can be used in outcrossing diploid species to drive population modification and suppression, suggesting possible applications in weed control, plant breeding and conservation.

Results

Components of a *ClvR*-based gamete killer.

The strength of a gene drive—its ability to spread from low frequency and in the presence of significant fitness costs—is increased when it biases inheritance in its favor in both sexes, something that is of particular importance when trying to bring about population suppression. Engineering *ClvR*-based gamete drive with this feature (Fig. 1a) requires targeting a gene whose expression during the haploid stage is required for the survival and/or development of microgametophyte and megagametophyte (hereafter referred to as pollen (which contains sperm) and ovules [the ovule being a sporophytic structure in the ovary within which each megagametophyte, which includes the egg, develops]). Mutations in many ubiquitously expressed housekeeping genes (such as were targeted for LOF allele creation in insect *ClvR*s[14,22]), likely have such a phenotype in plants given the extensive gene expression that occurs in gametes, but are challenging to identify since such mutations cannot be passaged through the germline. Their identity is sometimes inferred by their absence in mutant collections (e.g.[36]). Alternatively, with the advent of methods for CRISPR-based mutagenesis, genes whose mutation results loss of male and female gametes can be identified through reverse genetics approaches that incorporate a rescuing transgene into the mutagenized genetic background (e.g.[37,38]). Here we focus on one such gene, YKT61, a ubiquitously expressed R-SNARE protein involved in fusion between vesicle and target membranes[37].

The components that make up our *ClvR* gamete killers are illustrated in Fig. 1e. As a Rescue we utilized a genomic fragment containing the *Arabidopsis lyrata* YKT61 gene (in which some amino acid coding region differences were recoded back to those of *A. thaliana*, Extended Data Fig. 1). For the Cleaver, four gRNAs targeting conserved regions within the *A. thaliana* YKT61 coding sequence (see also Fig. 4) were expressed ubiquitously using individual U6 Pol-III promoters[39]. Several versions of Cas9 were tested. One lacks introns and carries a mutation (K918N) shown to increase Cas9 catalytic activity[40] while a second one contains 13 introns, which are thought to increase expression[41]. Regulatory sequences from six different genes were used to direct Cas9 expression. *Arabidopsis* DMC1 is primarily expressed during meiotic stages[42]. Sequences from the CLAVATA3 (early stem cell identity[43]), APETALA1 (flower meristem identity[44]) and AGAMOUS (reproductive floral organ primordia[45]) genes direct expression in adult sporophyte tissues that include the future germline. The CaMV35S[46] and UBIQUITIN10[47] promoters direct expression broadly, in many if not all cell types. The DMC1 promoter was used in combination with both versions of Cas9, while AGAMOUS, CLAVATA3, APETALA1, CaMV35S and UBIQUITIN10 sequences were used to direct expression of the version of Cas9 lacking introns.

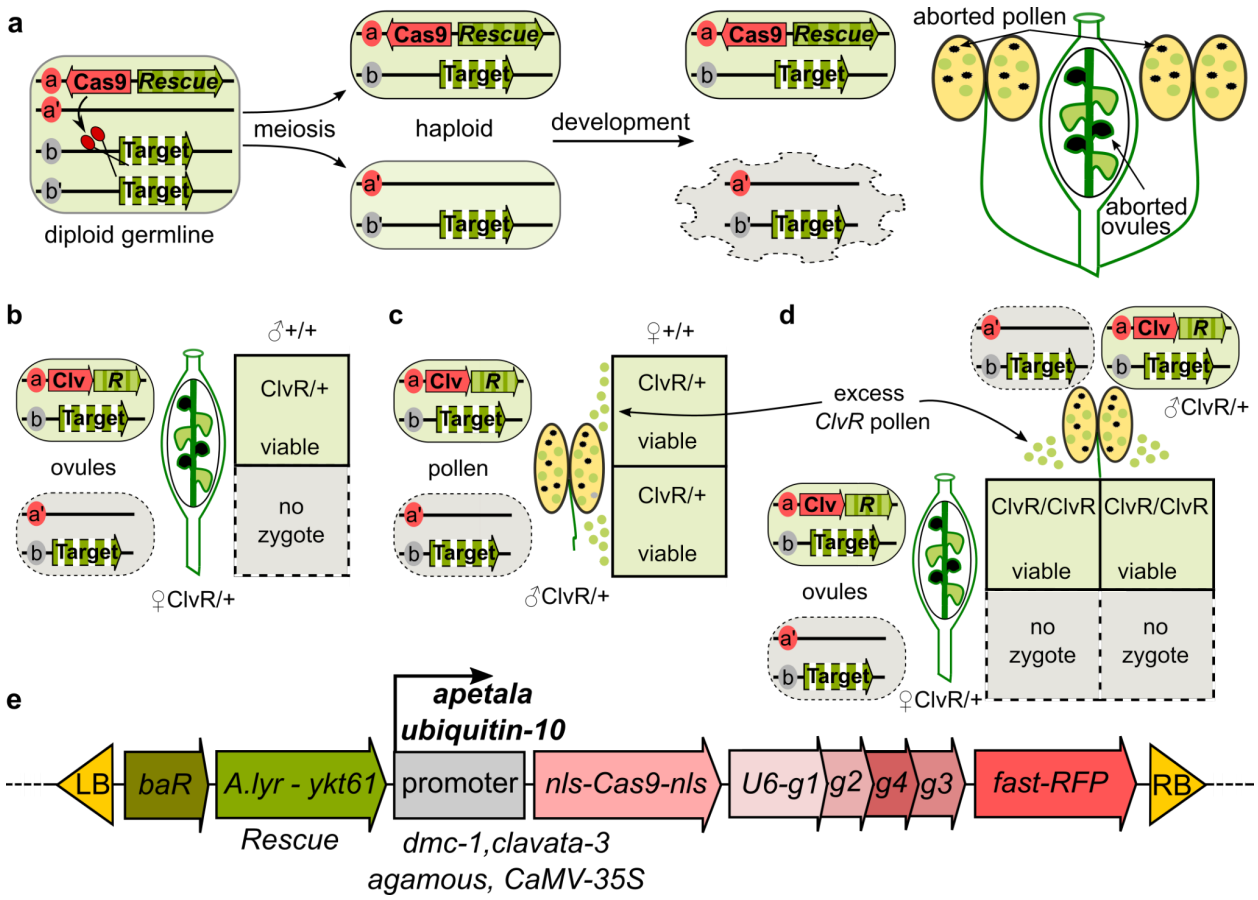

Fig. 1: *ClvR* behavior in a diploid plant and construct design. (a) Cas9/gRNAs located on chromosome **a** of an **a/a'** *ClvR* heterozygote cleave an essential gene located on chromosome **b** and **b'** during the diploid parental stage, creating LOF alleles. Diploid cells survive this because the *ClvR* carries a recoded rescuing version of the essential gene, which produces a functional product (light green background). During haploid stages expression of the essential gene is required for gamete/gametophyte development/survival. Gametes/gametophytes that fail to inherit *ClvR* lack a functional copy of the essential gene and die (indicated with dashed outline and gray background). The hermaphrodite plant to the right has anthers with *ClvR*-bearing pollen (green

circles) and dead non-*ClvR*-bearing pollen (dark circles) and an ovary containing *ClvR*-bearing ovules (large green shapes) and dead non-*ClvR*-bearing ovules (large dark circles). **(b)** Cross of a heterozygous *ClvR*-bearing female with WT (+/+) pollen. Non-*ClvR*-bearing gametophytes die and do not undergo fertilization (gray no zygote square). Thus, all progeny are *ClvR*-bearing heterozygotes (green square). **(c)** Cross in which pollen from a heterozygous *ClvR*-bearing male fertilizes ovules of a WT (+/+) female. Pollen is produced in large excess over ovules. Thus, death of the 50% non-*ClvR*-bearing pollen (dark circles) still allows all ovules to be fertilized, resulting in all progeny being *ClvR*-bearing heterozygotes. **(d)** Cross of a heterozygous *ClvR* female to a heterozygous *ClvR* male. Only *ClvR*-bearing ovules and pollen participate in fertilization, resulting in all progeny being homozygous *ClvR/ClvR*. **(e)** Genetic makeup of the *ClvR* drive element. From left to right these are: a Basta herbicide resistance marker (baR); a YKT61 rescue transgene derived from *Arabidopsis lyrata* (*A. lyrata* - YKT61); one of six different enhancer/promoters (those that resulted in significant transmission ratio distortion indicated in bold) used to direct Cas9 expression; Cas9 (one of two different versions, discussed in text); 4 gRNAs designed to base pair with DNA for the YKT61 coding region, with each expressed under the control of an independent U6 promoter (U6-g1-4); a fluorescent seed transgenesis marker (fast-RFP). Repeats required for transgenesis using agrobacterium (LB and RB) flank these elements.

***ClvR*s based on cleavage and rescue of the YKT61 gene show key features required for gamete killer gene drive**

We used floral dipping with agrobacterium to transform a number of T0 WT plants with the above constructs (Fig. 2a). A number of independent transformants, identified as red transgenic seeds of the T1 generation, were collected from these plants (Fig. 2b) and characterized in the crosses outlined in Fig. 2c-f. T1 seeds (heterozygous for one or more *ClvR* elements) were grown to adulthood and allowed to self (T1xT1; Fig. 2b). T1 self crosses that produced progeny siliques (a seed pod, which contains progeny from the ovules of one flower) containing all or primarily red seeds—the T2 generation, possibly *ClvR/ClvR* homozygotes (Fig. 2c)—were characterized further as this is the expected phenotype if gametic drive occurred in one or both sexes in a cross between heterozygotes (Fig. 1b-d). Based on the results of these experiments (a significant fraction of non-*ClvR* seeds), constructs utilizing regulatory sequences from the DMC1, AGAMOUS, and CLAVATA-3 genes were not considered further.

T2 seeds carrying constructs that utilized the APETELA1 and UBIQUITIN10 regulatory sequences were grown to adulthood and pollen from these plants was used in an outcross to WT to produce T3 *ClvR*/+ seeds (Fig. 2d). Finally, in the key outcross to test for gametic drive, T3 seeds were grown to adulthood and pollen and ovules used in outcrosses to WT (Fig. 2e). The frequency of *ClvR* inheritance (*ClvR/*+) in progeny T4 seeds provides a measure of gamete killing and rescue (Fig. 2f). *ClvR* inheritance rates in T4 seeds from T3 pollen, shown for 5 different insertions using UBIQUITIN10

sequences (*ClvR*^{ubq} lines) to direct Cas9 expression (Fig. 2h) were generally very high, with three of the five showing inheritance rates greater than 99%. Inheritance rates of *ClvR* in T4 seeds from T3 ovules were also significantly above 50%, but a number of non-*ClvR* seeds (generically referred to as escapers) were observed (Fig. 2g). Similar results were obtained for *ClvR*s utilizing APETALA1 regulatory sequences (Extended Data Fig. 2). Crosses with *ClvR*s using the CaMV35S promoter showed inheritance that was modestly *ClvR*-biased (Extended Data Fig. 3). These were not considered further. The basis for the differences between drive through pollen and ovules is discussed further below.

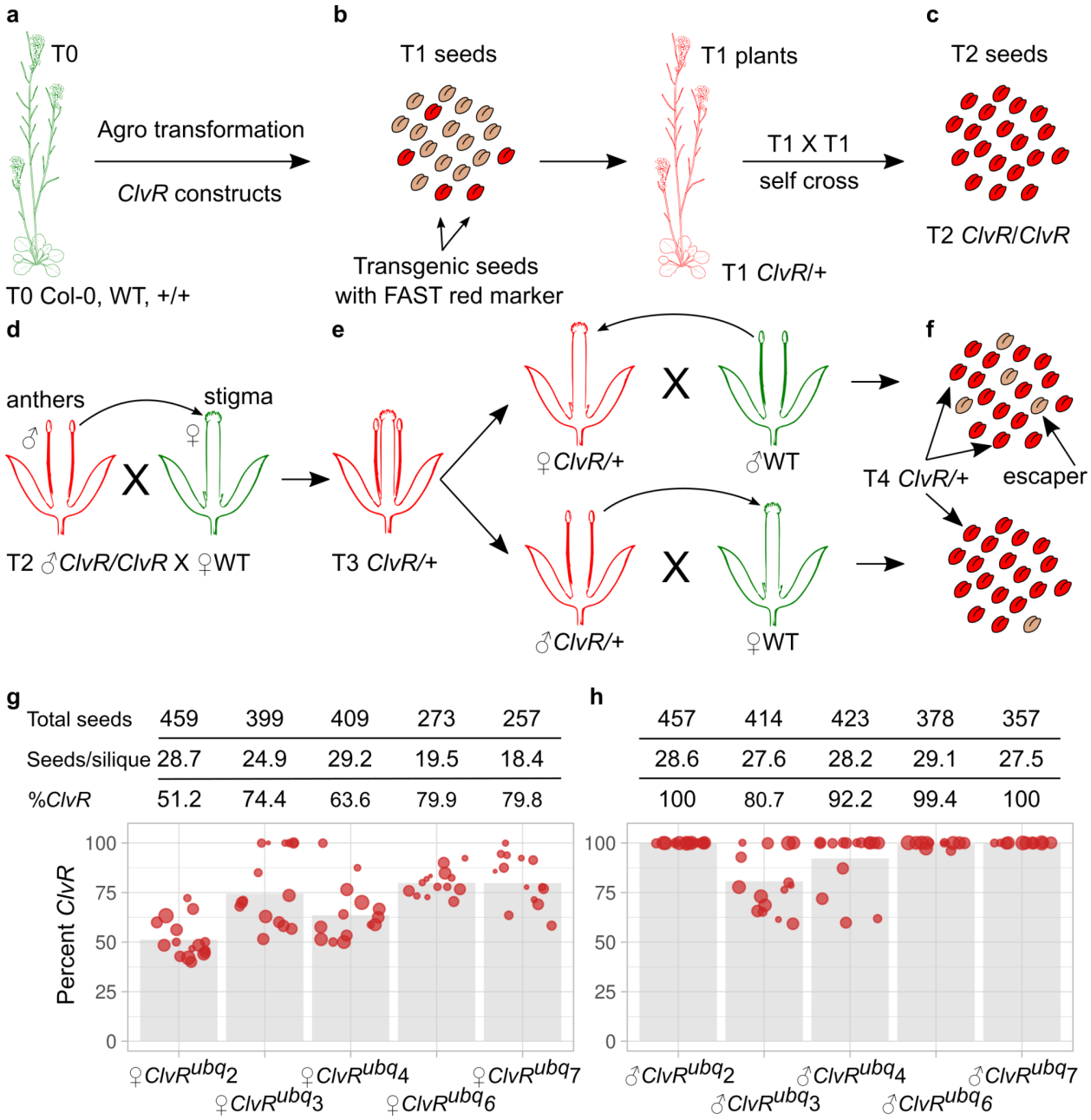

Fig. 2. Genetic evidence for *ClvR*-based gamete killing and rescue.

(a-f) Crosses used to establish independent *ClvR* insertions **(a-d)** and test for gametic drive through the male and female germline **(e-f)**. **(g,h)** Frequency of *ClvR* transmission through the female **(g)** and male **(h)** germline. We counted the total number of seeds and the number of seeds per silique. Each circle represents an individual silique. The

size of the red circle scales with the number of seeds in the silique. Note that for the female crosses in **g**, in general as the frequency of *ClvR* inheritance goes up, the number of seeds in the silique goes down. This is expected since the number of functional ovules determines the maximal seed output, and a *ClvR* with efficient killing and rescue would only be expected to produce half the WT number of functional ovules/seeds. Seed and silique counts are in Supplementary Table S1.

ClvR-based gamete killing and rescue is stable over multiple generations

The above results show that *ClvR*s designed to kill and rescue a gamete essential gene can bias inheritance in their favor in *Arabidopsis*, satisfying the key requirement for TA-based gametic gene drive. We focused our characterization on the *ClvR*^{ubq6} and *ClvR*^{ubq7} lines (generically referred to as *ClvR*^{ubq}) as these showed the highest frequency of inheritance through pollen and ovules, and heterozygotes and homozygotes were otherwise healthy (Extended Data Fig. 4). To determine if the bias in *ClvR*^{ubq} inheritance was stable, and if it had any dependence on the sex through which drive element was inherited, we characterized drive of ClvR^{ubq} alleles present in T4 *ClvR*^{ubq}/+ individuals (Fig. 2f) that came from either a male or female *ClvR*^{ubq}/+ T3 parent (Fig. 3). *ClvR*^{ubq}-bearing T4 seeds derived from male or female *ClvR*^{ubq}/+ T3 parents crossed to WT were grown to adulthood and pollen and ovules from *ClvR*^{ubq}-bearing T4 individuals were used in outcrosses to WT, giving rise to a T5 generation of seeds whose *ClvR*^{ubq}/+ grandparents (the T3 generation) and parents (the T4 generation) were either both female (ovules), both male (pollen), or one (T3) and then the other (T4) (Fig. 3a,b). As

shown in Fig. 3c,d inheritance rates remained comparable – very high when transmitted through pollen and high but with significantly more non-*ClvR*^{ubq}-bearing escaper seeds when transmitted through ovules – regardless of the parental and grandparental sex.

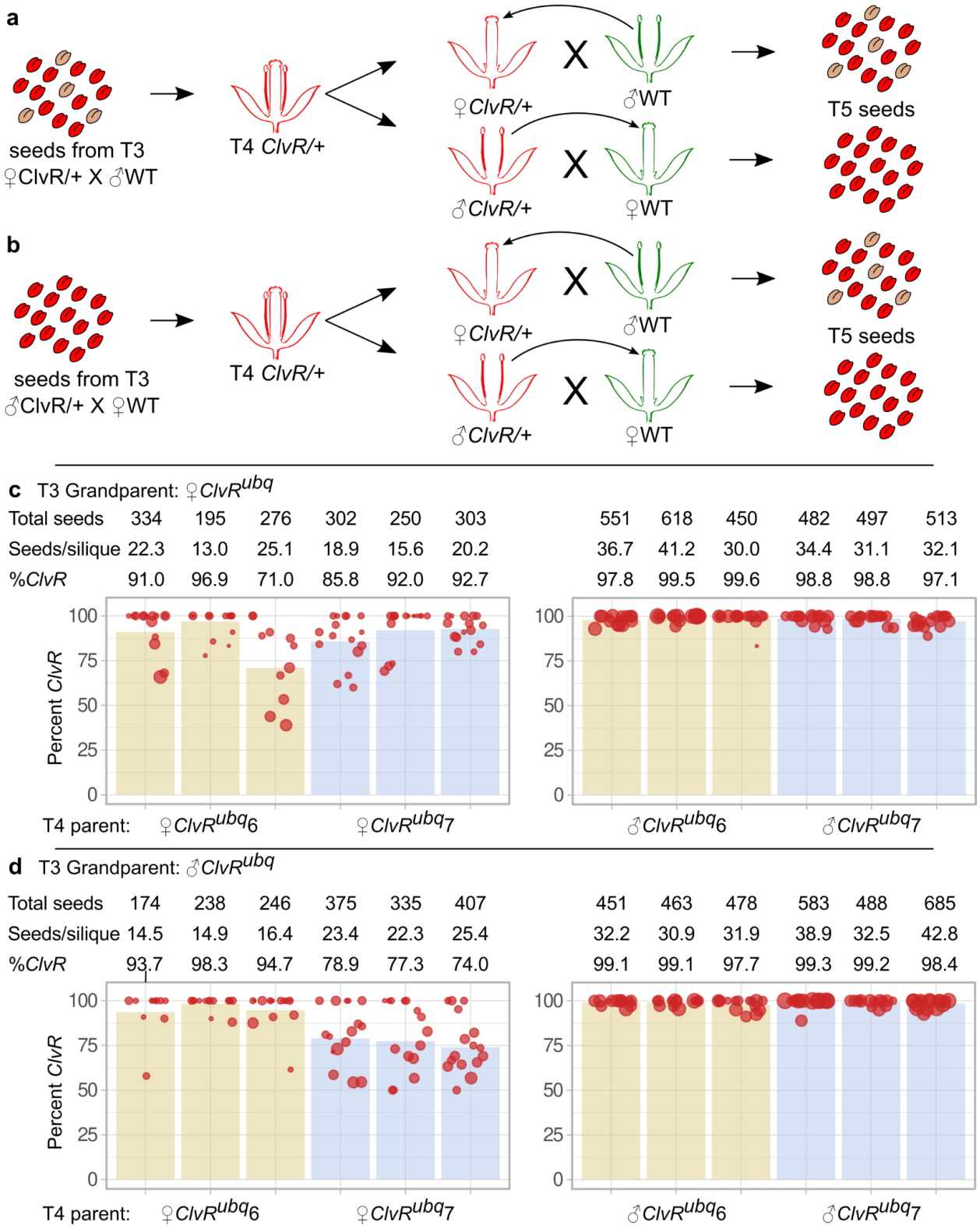
a
seeds from T3
♀ClvR/+ X ♂WT
T4 ClvR/+
♀ClvR/+
X
♂WT
T5 seeds
♂ClvR/+
X
♀WT
b
seeds from T3
♂ClvR/+ X ♀WT
T4 ClvR/+
♀ClvR/+
X
♂WT
T5 seeds
♂ClvR/+
X
♀WT
c T3 Grandparent: ♀ClvRubq
Total seeds 334 195 276 302 250 303 551 618 450 482 497 513
Seeds/silique 22.3 13.0 25.1 18.9 15.6 20.2 36.7 41.2 30.0 34.4 31.1 32.1
%ClvR 91.0 96.9 71.0 85.8 92.0 92.7 97.8 99.5 99.6 98.8 98.8 97.1
Percent ClvR
100
75
50
25
0
T4 parent: ♀ClvRubq6 ♀ClvRubq7 ♂ClvRubq6 ♂ClvRubq7
d T3 Grandparent: ♂ClvRubq
Total seeds 174 238 246 375 335 407 451 463 478 583 488 685
Seeds/silique 14.5 14.9 16.4 23.4 22.3 25.4 32.2 30.9 31.9 38.9 32.5 42.8
%ClvR 93.7 98.3 94.7 78.9 77.3 74.0 99.1 99.1 97.7 99.3 99.2 98.4
Percent ClvR
100
75
50
25
0
T4 parent: ♀ClvRubq6 ♀ClvRubq7 ♂ClvRubq6 ♂ClvRubq7

Fig. 3. *ClvR*-based gamete killing and rescue is stable over multiple generations. (a-b). (a) A T3 cross between *ClvR*^{ubq}/+ females and WT was used to generate T4 *ClvR*^{ubq}/+ heterozygous plants. Pollen and ovules from these T4 individuals were used in outcrosses to WT to generate a *ClvR*^{ubq} heterozygous T5 generation. **(b)** A T3 cross between *ClvR*^{ubq}/+ males and WT was used to generate T4 *ClvR*^{ubq}/+ heterozygous plants. Pollen and ovules from these T4 individuals were used in outcrosses to WT to generate a *ClvR*^{ubq} heterozygous T5 generation. **(c)** Frequency of *ClvR*^{ubq} inheritance in crosses in which the T3 grandparent was a *ClvR*^{ubq}/+ heterozygote female and T4 parents were either a *ClvR*^{ubq}/+ female (left six columns) or male (right six columns). **(d)** Frequency of *ClvR*^{ubq} inheritance in crosses in which the T3 grandparent was a *ClvR*^{ubq}/+ heterozygote male and T4 parents were either female (left six columns) or male (right six columns). Seed and silique counts are in Supplementary Table S1. A description of crosses is presented in Extended Data Fig. 5.

Mutations associated with cleavage and molecular basis of escape from gamete killing. The high frequency of *ClvR*^{ubq} inheritance when transmitted through *ClvR*^{ubq}/+ pollen argues that rates of cleavage and LOF mutation creation are high, and that rescue is efficient. The UBIQUITIN10 regulatory sequences drive expression broadly throughout development, from the embryo onwards[48], long before the male and female germlines form. This suggests that rates of cleavage and LOF allele creation in female gametes are high as well. To understand the molecular events associated with drive, and the unexpectedly high numbers of non-*ClvR*^{ubq} progeny observed when a *ClvR*^{ubq}/+

individual was the female parent, we sequenced the endogenous YKT61 locus in leaves of several genotypes: *ClvR*^{ubq}/+ T4 heterozygotes and *ClvR*^{ubq}/*ClvR*^{ubq} T5 homozygotes derived from a T4 self cross, with equal numbers of *ClvR*^{ubq6} and *ClvR*^{ubq7} sequenced for both crosses; and non-*ClvR*^{ubq} escapers from crosses of *ClvR*^{ubq}/+ to WT, in which the *ClvR*^{ubq}/+ was the female or male parent (Fig. 4). See Extended Data Table 1 for the details of sequence alterations at each gRNA target site. In *ClvR*^{ubq}/+ individuals all four target sites were cleavable and mutated to LOF (frameshifts) at high frequency, with at least three sites being altered in all 10 plants. In *ClvR*^{ubq}/*ClvR*^{ubq} homozygotes all four sites were altered in all 8 sequenced individuals. In outcrosses using *ClvR*^{ubq}/+ pollen a very small number of non-*ClvR*^{ubq} escaper seeds was observed (~1% of all seeds; Fig. 2 and Fig. 3). Twelve T4 escapers were grown to adulthood and sequenced. All were WT at all four gRNA target sites (Fig. 4). Thus, escape from death by non-*ClvR*^{ubq} pollen is due to lack of cleavage and/or sequence alteration following cleavage, at all four sites. In contrast, in crosses with females as the parent 18 out of 20 non-*ClvR* progeny of a *ClvR*^{ubq}/+ parent carried one or more sequence alterations at gRNA target sites that create LOF alleles (frameshifts) in the YKT61 coding region (Fig. 4 and Extended Data Table 1). The other two escapers were wildtype at all four target sites. No resistant versions of endogenous YKT61 — mutated but likely to be functional — were observed.

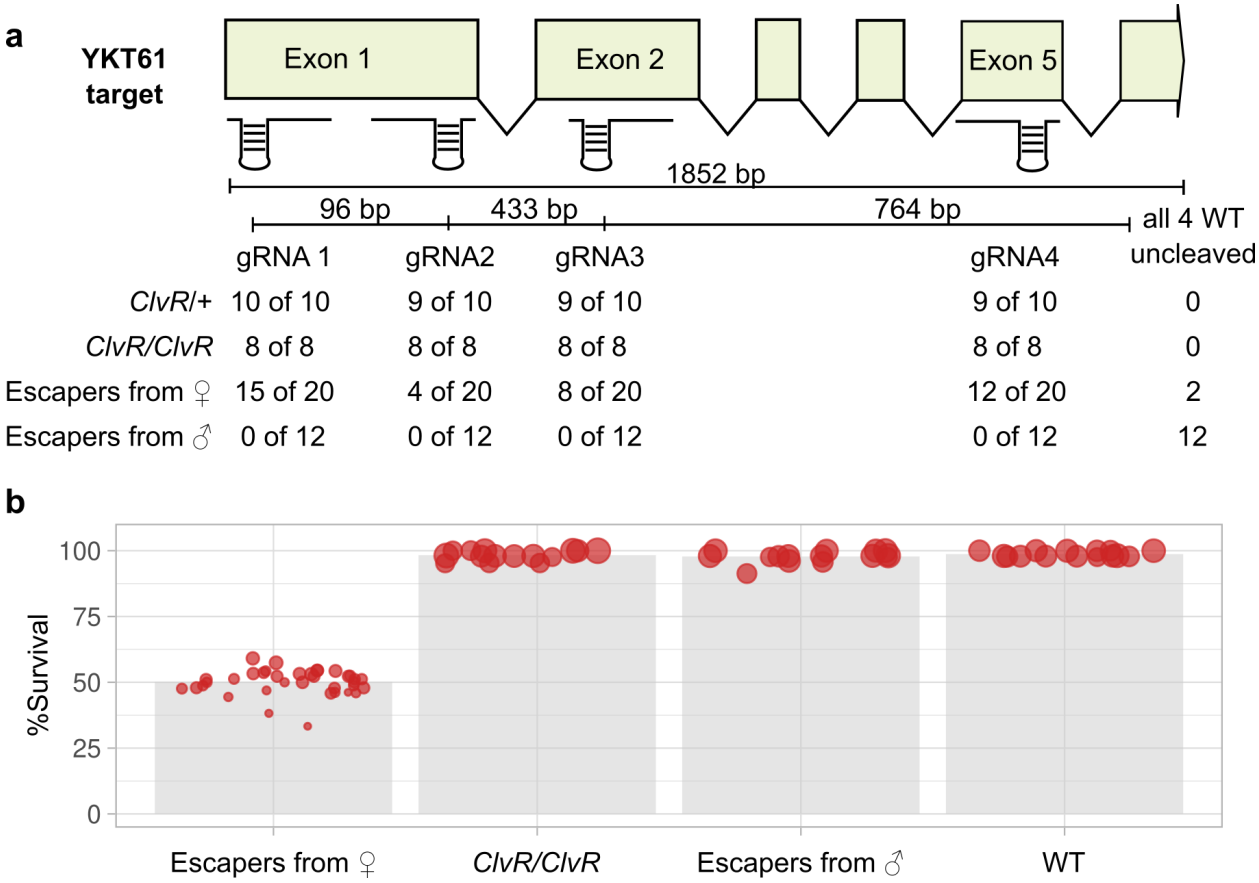

Fig. 4: Characterization of the target locus following exposure to *ClvR*^{ubq}, and genetic behavior of LOF mutations found in female escapers. (a) The genomic region containing the YKT61 gene is shown, along with the approximate locations of four gRNA target sites (see Extended Data Fig. 1 for exact sequences). The table summarizes how many of the target sites were altered to likely LOF (frameshifts or large deletions) in *ClvR*^{ubq}/+, *ClvR*^{ubq}/*ClvR*^{ubq} (heterozygous *ClvR*^{ubq}/+, inbred for 1 generation), escapers coming from a *ClvR*^{ubq}/+-bearing mother, and escapers from a *ClvR*^{ubq}-bearing father. 2 out of 20 escapers from *ClvR*^{ubq} mothers were WT at all four target sites. 12 out of 12 escapers from *ClvR*^{ubq} fathers were WT at all four target sites. **(b)** Fraction of total ovules in individual siliques (red circles) that developed into seeds in

self crosses from 4 different genotypes. Size of the circle scales with number of seeds in a silique. Escapers derived from a female *ClvR*^{ubq}/+ parent aborted ~50% of ovules, consistent with the known LOF phenotype of YKT61 mutants [37]. Escapers derived from a male *ClvR*^{ubq} parent (all WT at the YKT61 locus) showed high seed production as did *ClvR*^{ubq}/*ClvR*^{ubq} homozygotes.

To summarize, rates of cleavage and mutation to LOF at the YKT61 locus are very high in male and female gametes. In males death of non-*ClvR*^{ubq} pollen, coupled with efficient rescue of those inheriting *ClvR*^{ubq}, leads to a very high frequency of *ClvR*^{ubq} inheritance in progeny. In females inheritance of *ClvR*^{ubq} is also high, but there are also significant numbers of non-*ClvR*^{ubq} progeny. The results of sequencing show that most of these have a LOF mutation, probably created by *ClvR*^{ubq} at a much earlier stage in the diploid. The construct was not present in plants grown from escaper seeds (Extended Data Fig. 7), arguing against transgene silencing playing a role. In earlier work, Cas9-induced LOF alleles of YKT61 were uniformly not passed to progeny through female gametes, resulting in abortion of 50% of ovules in a self cross[37]. Given this, our observations suggest that in the *ClvR*^{ubq}/+ diploid the YKT61 rescue transgene from *A. lyrata* provides YKT61 transcript and/or protein that is carried over from the mother into the non-*ClvR*^{ubq} haploid ovules (maternal carryover rescue), and that this is sufficient to rescue the survival of some gametes carrying a LOF YKT61 allele. A strong prediction of this hypothesis is that LOF alleles generated by *ClvR*^{ubq} and present in LOF/+, non-*ClvR*^{ubq} heterozygotes should, since they lack the *ClvR*^{ubq} Rescue, not be

transmitted to the next generation. In a self cross of female escapers (most of whom are LOF/+ heterozygotes based on the results of sequencing; Fig. 4)) this should manifest itself as 50% abortion in progeny siliques. As illustrated in Fig. 4b and Extended Data Table 2 this is the phenotype we observed for a number of female escapers tested. In contrast, and as expected, the ovule abortion rate in self crosses of homozygous *ClvR*^{ubq}/*ClvR*^{ubq}, male escapers, and WT was very low.

The mechanism by which maternal *ClvR* rescues some female gametes from death requires further exploration. Recoding associated with use of *A. lyrata* YKT61 may have created an mRNA with an extended half-life. Alternatively, position effects based on chromatin structure and/or nearby transcriptional regulatory sequences may lead to increased expression and/or extend expression of *A. lyrata* YKT61 farther into meiosis, resulting in carryover into non-*ClvR* bearing gametes carrying a LOF mutation in YKT61. Incorporation of chromatin insulators[49] and/or targeting of other essential genes can minimize such effects.

Modeling predicts that *ClvR* elements can drive rapid population modification and suppression in plants.

To explore the utility of *ClvR*-based gamete killers for population modification and suppression we used a stochastic model (a panmictic population with non-overlapping generations that considers individual and gametes; see methods for details) to explore *ClvR* behavior in several scenarios. In animals, gene drive is often modeled using a

paradigm in which matings are monogamous and sperm is not limiting. However, in many plants of interest (crops, weeds, targets of conservation) polyandry (fertilization of a female with pollen sourced from multiple males, which is in excess) is likely to be more relevant[50]. In other contexts not explored here pollen limitation can occur[51]. The mating system is important to consider since the relative benefit in transmission frequency that *ClvR*-bearing gametes gain in fertilizing ovules, due to loss of competing non-*ClvR* gametes, decreases as the number of competing non-*ClvR* gametes from other males increases. Here we provide some representative examples of outcomes when a pan-gamete killing *ClvR* is introduced into a population and the mating system is monogamous — a best case scenario in which *ClvR* gametes from a single male monopolize the ovary of a female — or polyandrous, with 5 or 20 males each contributing 1/5th or 1/20th of their pollen to a female. We also consider the role of gamete fitness costs, as might arise due to incomplete rescue or cleavage-induced aneuploidy[52] that manifests itself as death during the haploid stage. The effects of maternal carryover of Rescue activity are also considered.

We first consider population modification. Fig. 5a-c shows examples in which *ClvR* is introduced at a frequency of 10% into a WT population. The LOF allele creation rate is set to 95%, somewhat lower than the rate inferred from the results of our experiments (Fig. 3 and Fig. 4). Gametic fitness costs (dominant because they are in the haploid stage) were varied between 0% and 15%. Maternal carryover was set to zero as exploration of other scenarios shows it has very little impact on population modification.

With monogamous mating *ClvR* spreads rapidly over a range of fitness costs (Fig. 5a). In the presence of polyandry drive is slowed and fails to spread for some higher fitness costs (Fig. 5b,c). However, drive can be restored if the introduction frequency is increased (Extended Data Fig. 6). When drive occurs, the *ClvR* spreads to allele fixation. This is because whenever a WT non-*ClvR* homologous chromosome is present with *ClvR* (and the LOF allele creation rate is high) it has a very high probability of being eliminated from the viable gamete pool since it lacks a Rescue transgene. Strong but self-limiting drive can presumably also be achieved by locating Cas9 (or gRNAs) at some distance from the other *ClvR* components, as with *ClvR*s that kill specific zygote genotypes[23].

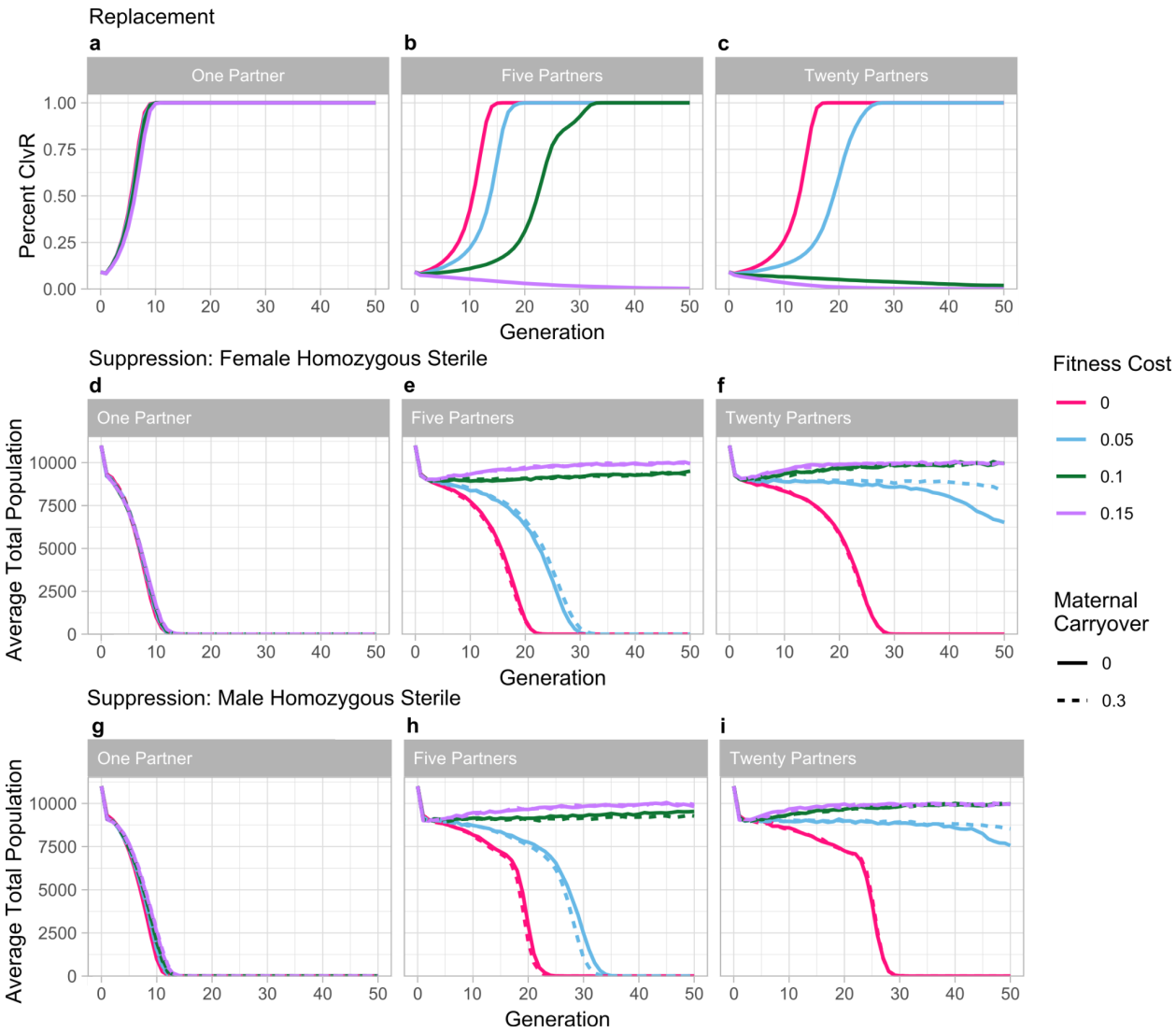

Fig. 5: Predicted behavior of *ClvR* for population modification and suppression. (a-c) Population modification. *ClvR* is introduced as homozygous males at a frequency of 10% of the starting population, which is at carrying capacity, 10,000 individuals. The mating system is monogamous **(a)**, or polyandrous, with 5 males each providing 1/5th of the sperm needed to fertilize all ovules of an individual female **(b)**, or 20 males each providing 1/20th of the sperm needed **(c)**. Fitness costs are incurred by gametes (a probability of not being able to participate in fertilization, if chosen by the model).

Maternal carryover is set to zero. Lines represent the average of 10 runs. **(d-f)** Population suppression with a transgene inserted into a recessive locus required for female sporophyte fertility. *ClvR* is introduced as above, at a frequency of 10%. The mating system is monogamous **(d)**, or polyandrous, with 5 males each providing 1/5th of the sperm needed to fertilize all ovules of an individual female **(e)**, or 20 males each providing 1/20th of the sperm needed **(f)**. Fitness costs are as above. Maternal carryover is set to zero or 30% (the approximate value observed in our experiments with *ClvR*^{ubq}). **(g-i)**. As with **d-f**, but with the *ClvR* inserted into a locus required for male sporophyte fertility. For these simulations homozygous females were released into the population since homozygous males are sterile. Lines represent the average of 10 runs.

For population suppression we consider two scenarios, in which *ClvR* is located in a gene (thereby disrupting it) whose recessive LOF in the sporophyte results in female (Fig. 5d-f) or male (Fig. 5g-i) infertility[16]. As above, gamete killing and rescue occurs in both sexes and some level of maternal carryover rescue of LOF allele-bearing gametes may be present. In both scenarios a gamete killer can drive the population towards a homozygous male or female sterile state, resulting in population extinction. Maternal carryover has a very modest effect on drive towards a homozygous female sterile state because it allows some WT alleles to persist for some time in non-*ClvR* progeny of a *ClvR*-bearing mother. However, it has no effect on drive towards a homozygous male sterile state. As with population modification, fitness costs can slow or prevent drive and

suppression, but increasing the introduction frequency can restore drive to the homozygous sterile state (Extended data Fig. 6).

Discussion

Our results argue that gamete killers based on a *Cleave* and *Rescue* mechanism provide a general strategy for bringing about gene drive-mediated population modification or suppression in diploid plants. *ClvR* elements utilize a simple toolkit of components that should be available in many species: a site-specific DNA-modifying enzyme such as Cas9 and the gRNAs that guide it to specific targets, sequences sufficient to direct gene expression in cells that will become the germline (which, as in our work, need not be germline-specific), an essential gene to act as target, and a recoded version of the essential gene resistant to sequence modification and able to rescue the LOF condition. For population modification these components can be located at sites distant from the target essential gene. For several strategies for population suppression the element needs to be located in a gene whose recessive LOF in the sporophyte results in either male or female infertility. Many such genes are known[53–56], particularly for male fertility. Alternatively, if LOF alleles of the sporophyte fertility gene can be efficiently created late in germline development, after the time when gene function is needed for fertility, then the element can be located anywhere in the genome. In this latter configuration Cas9/gRNAs and Rescue bring about gametic drive towards *ClvR* homozygosity, while cleavage and LOF allele creation in the fertility gene during

meiosis (which could use an orthogonal RNA-guided DNA sequence modifier) ensures that all homozygous progeny (males or females), but not heterozygotes, are sterile.

In the context of suppression, we also note that if a gamete killing *ClvR* (or a killer of non-*ClvR* gametes in only pollen) is tightly linked to genes that are sufficient for male sex determination (e.g.[57,58]) then *ClvR*-bearing individuals (by definition males) will only produce *ClvR*-bearing sons and pollen that gives rise to male progeny. Modeling and experiments focused on similar Y-linked killers of X-chromosome-bearing sperm in animals shows that such a system can be used to drive population suppression when females mate with one or a few males[8,59]. As discussed in the context of Fig. 5, drive towards population elimination becomes weaker as the level of polyandry increases, but may still occur in a timely manner if the introduction frequency is increased.

Finally, the *Cleave* and *Rescue* mechanism could also be used in a non-gene drive strategy for population suppression. This involves creating an autosomal female sterile *ClvR* ($ClvR^{FS}$) in which males produce fertile *ClvR*-bearing males (which produce only *ClvR*-bearing pollen) and sterile females. Modeling and experiments in insect systems show that periodic releases of males with these features (only male progeny of a male carrier are viable or fertile) can bring about population suppression or elimination by driving a progressive decrease in the number of fertile females[60–62]. Such an element does not show self-sustaining drive because it finds itself in dead-end females half the time. However, its persistence over multiple generations in fertile males provides an

ongoing force that contributes to a reduction in the number of fertile females. *ClvR*^{FS} can be created in several ways. First, a pan gamete killing *ClvR* can be located within (thereby disrupting) a gene whose expression in female gametes is required for their survival. Males carrying this construct produce *ClvR*-bearing pollen. Sons are fertile and pass on only *ClvR*-bearing pollen. In contrast, daughters that inherit *ClvR* are sterile. Those female gametes that inherit the *ClvR* die because they lack the female gamete essential gene while those that lack *ClvR* die because they lack a functional copy of the pan gamete essential gene targeted for LOF allele creation by *ClvR*. A similar effect can be achieved by linking a pan gamete *ClvR* located at a neutral position with a transgene that dominantly blocks female fertility.

Our experiments focused on cleavage and rescue of the ubiquitously expressed R-SNARE YKT61 gene. It is likely that many other ubiquitously expressed housekeeping genes can be targeted to similar effect. Alternatively, drive can be limited to one sex or the other by targeting genes required more specifically for gametogenesis in only one sex (e.g.[30,31]. Ideally Cas9 expression, cleavage and LOF allele creation would be limited to cells of the appropriate reproductive organ or meiosis, so as to minimize fitness costs associated with Cas9 expression or heterozygosity for LOF mutations in the target gene (haploinsufficiency), and allow for targeting of fertility genes after the time in development they are needed. These were the reasons we tested regulatory sequences from genes with restricted expression patterns that include the future germline: DMC1 and APETELA1, CLAVATA3 and AGAMOUS. Among these only

APETELA1 sequences showed evidence of strong drive in males. Low levels of drive were observed in females. Based on the results of experiments discussed above we speculate this may be because of particularly strong maternal carryover rescue. Even when using the UBIQUITIN10 sequences to drive Cas9 expression, providing many opportunities throughout development to cut and create LOF alleles, we observed a low frequency (~1%) of uncut/unmodified alleles at the YKT61 locus in escapers. This is not due to transgene silencing since the construct was not present in escaper seeds (Extended Data Fig. 7). Nucleosome structure has been shown to inhibit Cas9 cleavage efficiency[63–65], and could play a role, though it is surprising that all four sites remained uncleaved. Only the results of more experiments with diverse promoters, and other RNA-targeted DNA sequence modifying enzymes that cleave or bring about base editing, acting on YKT61 and other target genes, will provide guidance on how best to ensure that all target sites are modified. Regardless, our modeling shows that a low frequency of WT escapers (which are still subject to cleavage in future generations) does not prevent population modification or suppression.

The modeling results presented in Fig. 5 show that *ClvR* gamete killers can bring about population modification and suppression under a variety of conditions. Fitness costs, as with other TA-based drive systems that target specific zygote genotypes for elimination, can slow or prevent drive, as can polyandry (Fig. 5), while increasing the introduction frequency supports drive (Extended Data Fig. 6). These conclusions notwithstanding, the type of model used—with a panmictic population and non-overlapping

generations—provides only heuristic guidance and is not predictive for any particular species or environment, since it does not include consideration of many other environment and species-specific variables. These include the mating system, overlapping generations (including the presence of seed banks), spatial structure, pollen and seed flow throughout that structure, whether pollen is in excess or limiting for fertilization, and the details of density dependence. Temperature sensitivity of DNA sequence modifying enzymes such as Cas9 and how this interfaces with climate and the timing of cleavage and gamete development will also be important to consider.

Our experiments and modeling focused on *Arabidopsis thaliana*, a diploid with a relatively small genome. Many plants of interest are polyploid[66]. Large genomes and polyploidy create several challenges. First, large genome size means Cas9 must sample a much larger genomic sequence space in a timely manner[67], which will require increased expression levels or the use of variants with increased catalytic activity. Second, polyploidy may release duplicated genes, even those encoding highly conserved housekeeping genes, from selective pressures that constrain their coding sequence, making it more difficult to identify gRNA target sites that remain unchanged. The design of gRNAs will be particularly challenging in allopolyploids, which have two or more complete sets of chromosomes from different species. The gene dosage needed for rescue (1 or multiple copies) also needs to be explored for polyploids. Suppression mechanisms that require insertion of *ClvR* into a gene required for gamete function may be challenging for related reasons. Finally, we note that plants often have a much

greater ability to hybridize across species barriers than do animals[66,68]. Thus, drive in some plants may lead to spread in wild relatives—though sequence divergence at gRNA target sites, if the gRNAs are chosen to be target species specific, can prevent this. In short, much remains to be explored as to species and contexts in which the key mechanisms required for *ClvR*-based gene drive (high frequency creation of LOF alleles and rescue) are most likely to be efficient and evolutionarily robust, and in which gene flow can be managed.

The above-noted unknowns notwithstanding, there are a number of possible applications of *ClvR*-type gene drive in plants. Ecosystem conservation is one use. This can take the form of suppressing invasive species. Alternatively, population modification can be used to engineer pathogen resistance or other forms of resilience (a form of genetic rescue) into native species in the face of novel stresses such as climate change. In the context of agriculture gene drive can be used to spread desirable agronomic traits. It has also been discussed as a possible tool for weed management[69,70]. Several approaches, which are not mutually exclusive, have been proposed. The first is direct population suppression. The second is sensitization, in which the goal is to drive a trait into the population that makes it less fit in a managed agricultural environment or specifically sensitive to some other intervention, such as herbicide application. One possible target species for both approaches is *Amaranthus palmeri* (Palmer amaranth), an invasive agricultural weed that is very economically destructive and difficult to manage[71]. Features that make Palmer amaranth amenable to

gene drive-mediated suppression and/or sensitization are that it is an annual, dioecious (plants are either male or female), a diploid, and a region containing genes associated with male sex determination has been identified[57]. In many locations Palmer amaranth has become resistant to currently available herbicides, with a key source of resistance (and thus a good target) being a large autonomously replicating extrachromosomal circular DNA transmitted through pollen[72,73].

Finally, the Cleave and Rescue mechanism may also have other applications in plant breeding not directly related to gene drive. Gamete killing and rescue can be combined with other activities to guide and limit the genotypes and phenotypes of progeny produced. For example, if a *ClvR* is brought into a maternal genetic background that results in cytoplasmic male sterility, *ClvR*-bearing females will produce only fertile *ClvR*-bearing females (non-*ClvR* female gametes are killed) and sterile males. A similar outcome (*ClvR* passed through one sex only) can be achieved by linking *ClvR* with a dominant transgene that acts in the sporophyte stage to eliminate female or male gametes. Movement of *ClvR*-linked transgenes through one sex only can also be ensured if the *ClvR* is located within, and disrupts, a gene required for sex-specific gamete survival, as discussed above. In each of the above cases gene drive (spread to a higher frequency) does not occur because the *ClvR* finds itself half the time in a dead-end sex from which it cannot be transmitted to the next generation. Maternal or paternal carryover of active Cas9/gRNAs into the zygote, as happens with *ClvR* gene drive in insects, and which can probably be engineered in plants using late gametophyte

promoters, provides another mechanism by which genotypes and phenotypes of offspring can be dominantly altered.

Methods

Synthesis of *Arabidopsis ClvR* constructs

In this study, constructs were assembled using Gibson cloning[74]. The gRNA cassette, composed of four repeats of gRNA with U6 promoters, was cloned with Golden Gate assembly. Enzymes utilized were obtained from NEB, and cloning as well as DNA extraction kits were sourced from Zymo. The *A. lyrata* Rescue gene was synthesized by Twist Bioscience.

We began with an intronized Cas9 variant known as zCas9i (pAGM55285, which was a gift from Sylvestre Marillonnet, Addgene #153212)[75]. In this Cas9 version, we replaced the RPS5 promoter with that of DMC1. Additionally, immediately upstream of the start codon, we incorporated 21 base pairs from AT1G58420, which had previously been demonstrated to enhance translational efficiency[76].

Finally, we integrated the recoded *A. lyrata* Rescue into the construct. A detailed sequence map is provided in Supplementary file 1.

gRNA design and cloning

To assemble the gRNA cassette we used the shuttle vectors from Stuttmann et al (pDGE332, pDGE333, pDGE335, and pDGE336, which were a gift from Johannes

Stuttmann, Addgene #153241, 153242, 153243, and 153244)[77]. Guides were designed in Benchling to target exon 1 (gRNA1 and 2), exon 2 (gRNA3) and exon 5 (gRNA4) of YKT61 and cloned into the Bbs1 digested shuttle vectors with annealed primers. The final Golden Gate assembly was performed with the Cas9-Rescue plasmid from above and Bsa1.

Cas9 promoters

We could not detect any cleavage with the intronized Cas9 and the DMC1 promoter, as inferred by the Mendelian inheritance of the full *ClvR* construct in multiple transgenic lines. Based on these results we replaced the intronized Cas9 with one that had no introns but retained the NLS sequences at the termini (Cas9 without introns from pTX168, which was a gift from Daniel Voytas, Addgene #89257)[78]. Additionally, we introduced a mutation (K918N) in the Cas9 sequence that was shown to enhance its catalytic activity[79]. However, the DMC1-Cas9 version without introns also showed no evidence of cleavage. Based on the results obtained with other promoters (APETELA and UBIQUITIN 10) and this version of Cas9, we inferred that the DMC1 promoter is likely to be relatively weak. For all additional promoters tested here we used the version of Cas9 version lacking introns and carrying the K918N mutation.

Using Gibson assembly, we replaced the DMC1 promoter with transcriptional regulatory sequences from APETALA1, CLAVATA*3*, and AGAMOUS (chosen based on their efficacy in previous work utilizing a Cre/Lox reporter)[80]. Finally, we also built two

versions of *ClvR* utilizing regulatory sequences that drive more ubiquitous expression, from the UBIQUITIN10 gene and the CaMV35S promoter. Whole plasmid sequencing was performed by Plasmidsaurus, using Oxford Nanopore Technology with custom analysis and annotation. Genbank files of all *ClvR* constructs with attached Gibson cloning and sequencing primers utilized in this study are in Supplementary File 1.

Arabidopsis handling

All plants in this study were grown in soil with a 16/8 hour light dark cycle. Temperature was 25℃. All seeds were planted directly in soil and stratified at 4C for 3 days. Transgenic plants were maintained in separate dedicated room in a hood mounted with a screen and surrounded by sticky tape, to minimize airflow around the plants and to prevent potential insect-mediated pollen movement. A floor-mounted sticky surface surrounding the hood performed a similar function. Transgenic plants were disposed of following autoclaving.

Arabidopsis Transgenesis

We used the floral dip method with agrobacteria as described previously[81]. *ClvR* plasmids were transformed into GV3101 ElectroCompetent Agrobacterium strain from Intact Genomics. T1 seeds were screened for the FAST red seed marker[82] and planted as described above.

Crosses to determine *ClvR* drive activity

Red T1 seeds were grown and allowed to self cross. Siliques (seed pods, with each pod representing the fertilized ovules of a single ovary/flower) of these plants were screened for the FAST red marker again. We looked for plants that showed 100% *ClvR* bearing seeds, suggesting drive activity (Mendelian genetics would result in 75% red seeds). At this stage we saw that *ClvR*^{dmc1}, *ClvR*^{agamous}, *ClvR*^{clavata3} had less than 100% red seeds in the self crosses and decided not to further characterize these lines.

T2 *ClvR* seeds were grown and pollen from these plants was used in an outcross to WT females to generate heterozygous *ClvR/+* T3 seeds. T3 seeds were grown into adults again to set up reciprocal crosses with WT. ♀*ClvR*/+ for each line were crossed to ♂WT and ♂*ClvR*/+ were crossed to ♀WT (4 crosses per plant, 4 plants per line). Siliques of these crosses were scored for the *ClvR* marker (results in Fig. 2).

Next, we took T4 seeds from individual T3 crosses and repeated a set of reciprocal (male *ClvR/+* to female WT and female *ClvR/+* to male WT) crosses. We crossed 4 plants with 4 crosses per plant for each of 3 individual T3 crosses (results in Fig. 3). We also collected leaf tissue from T4 heterozygous *ClvR* plants and escapers from the ♀*ClvR*/+ X ♂WT cross (non-*ClvR* bearing seeds) to extract DNA and sequence the YKT61 target sites (see below). Sequencing results are in Extended Data Table 1. Finally, we grew T5 escaper seeds coming from ♀*ClvR*/+ and from ♂*ClvR*/+. Leaves of young plants were again collected, and target sites sequenced as described below.

Molecular analysis of cleavage events

DNA from candidate plants was extracted from leaves with the Zymo Quick-DNA Plant/Seed miniprep kit according to the manufacturers protocol. The YKT61 target region was PCR amplified using primers ykt-cleaveF1 (TAGCATCTCCGAGTAAGGAATC) and ykt-cleaveR2 (CTTATAGATTTAGTTTCCTTTTTTCCCTGT). The PCR fragment was purified following agarose gel electrophoresis and sequenced by Plasmidsaurus at ~1000X coverage. The resulting raw reads were mapped to the YKT61 reference using minimap2[83]. The alignment file was sorted and indexed with samtools. The output file variants were then clustered with a Python script from Pacific Biosciences (https://github.com/PacificBiosciences/pbampliconclustering). Mutations were analyzed in the output "variantFraction" file. Results are summarized in Extended Data Table 1.

Modeling

Modeling was performed using a stochastic agent-based model with discrete generations written in python. The model tracked and recorded dioecious diploid individuals in each generation and tracked gametes between generations (explained in Extended Data Fig. 8). For the data shown here (Fig. 5 and Extended Data Fig. 6), we assumed *ClvR* and the target gene were unlinked, that our *ClvR* element had 95% efficiency in creating LOF alleles, and that the population had a low-density growth rate of 6. More information on the model, the scripts and parameters used to generate the data, and the data itself can be found on https://github.com/MicycleJ/Pigss/tree/main

Data Availability

All data is available in the main text or the supplementary information files.

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Contributions

Conceptualization, G.O., T.I. and B.A.H.; Methodology, G.O., T.I., M.L.J., and B.A.H.; Investigation, G.O., M.L.J, B.A.H.; Writing – Original Draft, G.O. and B.A.H.; Writing – Review & Editing, G.O., T.I., M.L.J. and B.A.H.; Funding Acquisition, B.A.H.

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Ethics declaration

The authors have filed patent applications on *ClvR* and related technologies (U.S. Application No. 15/970,728 and No. 16/673,823).

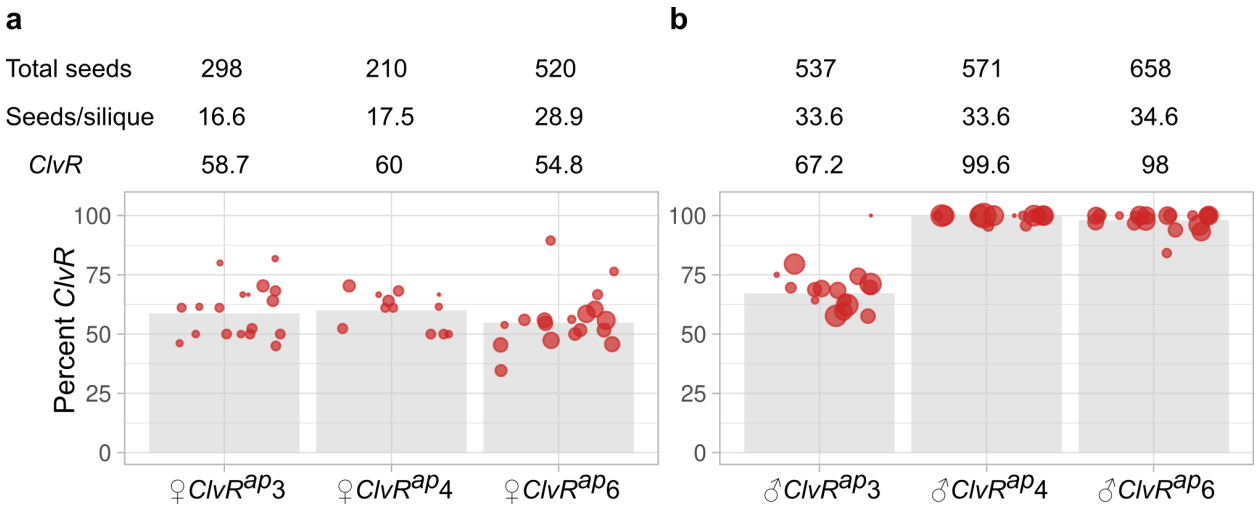
Data availability

All data is available in the main text or the supplementary materials.

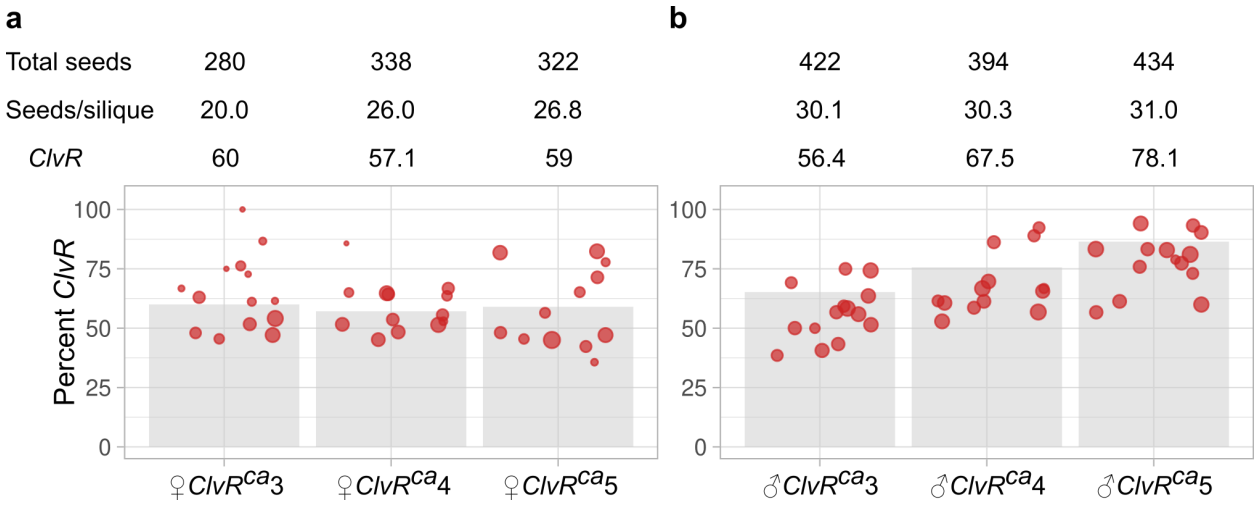
Extended Data Fig. 1-8
Extended Data Table 1-2

```
Alyr-recoded    atgaagatcacTGCTTTGTTGGTTTTGAAGTGTgctcccgaaGcttctgatcccgttatc  60
Athal           ATGAAGATCACCGCCTTGCTCGTTCTCAAGTGCGCTCCCGAAGCTTCTGATCCCGTCATC  60
                *********** ** *** * *** * ***** ********************** ***
                                      gRNA1
Alyr-recoded    ctcTCTaacgcttccgacgtctctcacttcggtTATTTTCAAAGATCTTCTGTTAAAGAA  120
Athal           CTCTCCAACGCTTCGGACGTCTCTCACTTCGGTTATTTCCAGCGATCTAGCGTCAAGGAG  120
                ***** ******** ********************* **.*****:  ** **.**.
                                                    gRNA2
Alyr-recoded    ttcgtcgtctttgtcGGAAGAACTGTTGCTTCTAGAACTCCTCCTTCTCAAcgccagtct  180
Athal           TTCGTCGTCTTTGTCGGTCGCACTGTCGCTAGCCGAACCCCTCCTTCCCAACGCCAGTCT  180
                *****************:.*.***** ***:  .**** ******** ************

Alyr-recoded    gtccaacacgaagagtacaaggttcacgcttacaatagaaatggcctttgcgcggtgggg  240
Athal           GTTCAACACGAAGAGTACAAGGTTCACGCTTACAATAGAAATGGCCTTTGCGCGGTGGGT  240
                ** ******************************************************

Alyr-recoded    ttcatggatgatcattatcctGTTAGATCTGCTTTTTCTcttcttaatcaggtcctagat  300
Athal           TTCATGGACGATCATTACCCTGTTCGAAGTGCTTTCTCTCTTCTCAATCAGGTCCTAGAT  300
                ******** ******** ******.**: ****** ******** ***************
                                      gRNA3
Alyr-recoded    gagtaccagaagagttttggtgagTCTtggaggtctgcaaaagaagactccaAtcagcct  360
Athal           GAGTACCAGAAGAGTTTTGGTGAGTCATGGAGGTCTGCAAAAGAAGACTCCAATCAGCCT  360
                ***************************:*********************************

Alyr-recoded    tggccatacttaactgaagctttaaacaaatttcaggacccagcagaggctgataagctg  420
Athal           TGGCCATACTTAACCGAAGCTTTAAACAAATTTCAGGACCCAGCAGAGGCTGATAAGCTG  420
                ************** *********************************************

Alyr-recoded    ttgaaaatccagagggagttggatgaAaccaagattatccttcataaaaccattgatagt  480
Athal           TTGAAAATCCAGAGGGAGTTGGATGAGACAAAGATTATCCTTCATAAAACCATTGATAGT  480
                **************************.**.******************************

Alyr-recoded    gttctagcccgtGGAGAAAAGTTGGATTCTTTGGTTGAAaagagctcagatttaagcatg  540
Athal           GTTCTAGCCCGTGGTGAGAAGCTGGACAGCCTAGTGGAGAAGAGCTCAGATTTGAGCATG  540
                **************:**.*** **** :   *.** **.**************.******
                                      gRNA4
Alyr-recoded    gcatcacagatgttttacaagcaagcgaagaaaacaaattcatgctgtactattctgtga  600
Athal           GCATCACAGATGTTTTACAAGCAAGCGAAGAAAACAAATTCATGCTGTACTATTCTGTGA  600
                ************************************************************
```

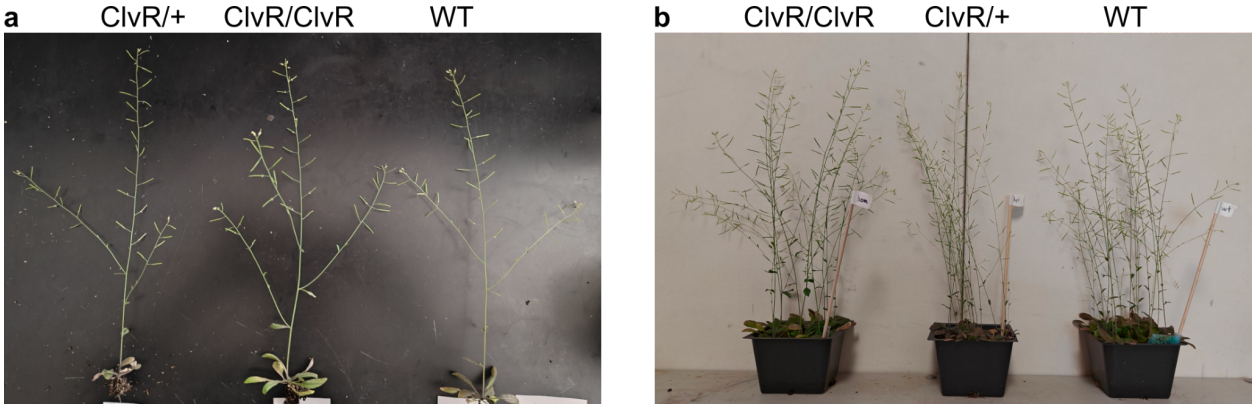
Extended Data Fig. 1: Alignment of the recoded *A. lyrata* rescue coding region to the *A. thaliana* target. Guides are indicated as red arrows. Note that the full sequence of the *A. lyrata* YKT61 genomic region used for rescue (Supplemental File 1) contains many additional differences from the equivalent *A. thaliana* sequence, in regulatory sequences, introns and 5' and 3' UTR. The amino acid sequences of the two proteins are identical.



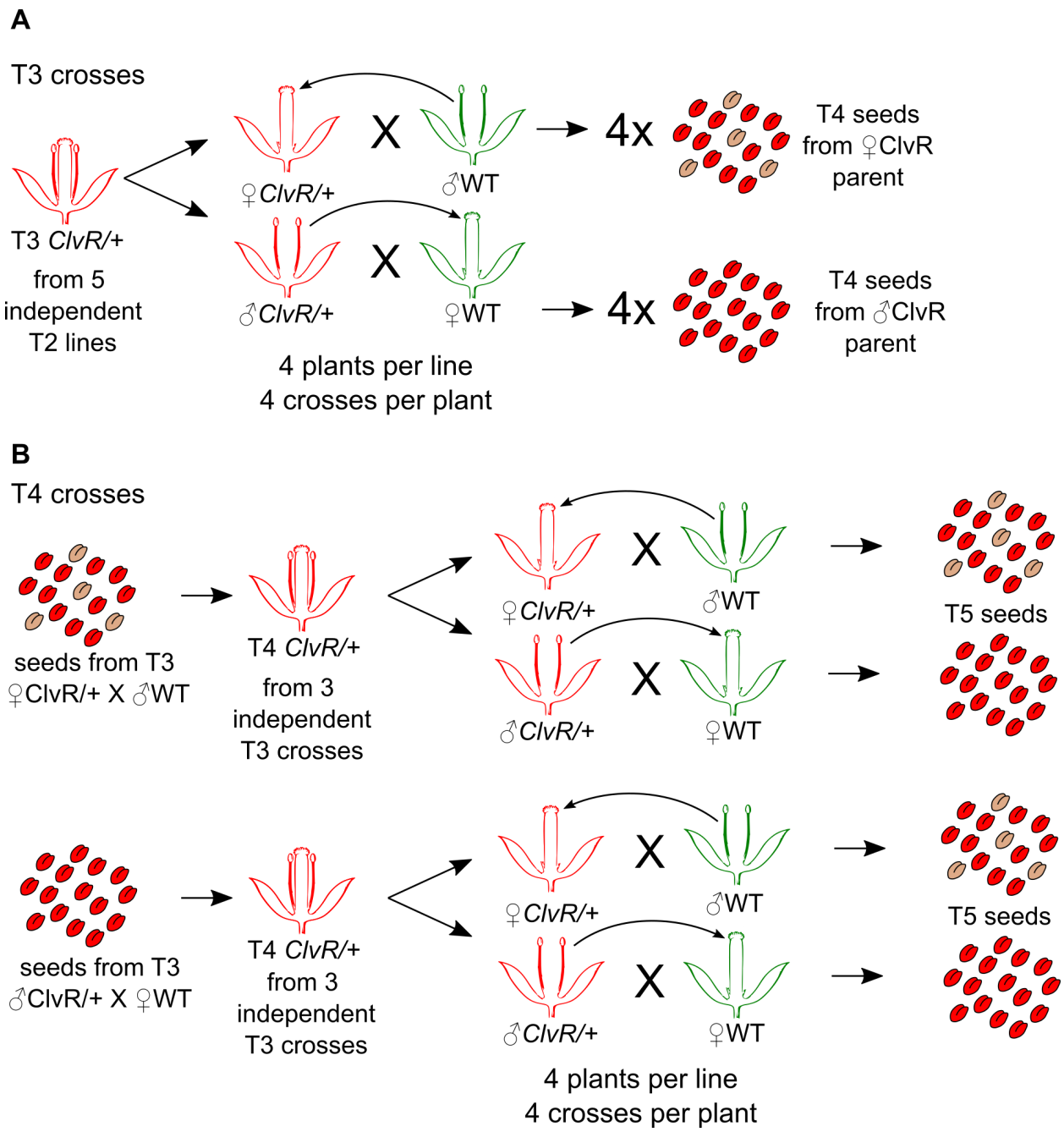
Extended Data Fig. 2: T3 heterozygous *ClvR* crosses for (a) female *ClvR*^{ap} and (b) male *ClvR*^{ap}. T3 *ClvR*^{ap}/+ heterozygotes were grown to adulthood and their ovules (left three columns) or pollen (right three columns) used in outcrosses to WT. Bar graphs show the number of siliques scored (red circles) and the percent *ClvR* seeds produced in the T5 generation. The number of seeds within each silique scales with circle size. Counts are in Supplementary Table S2.



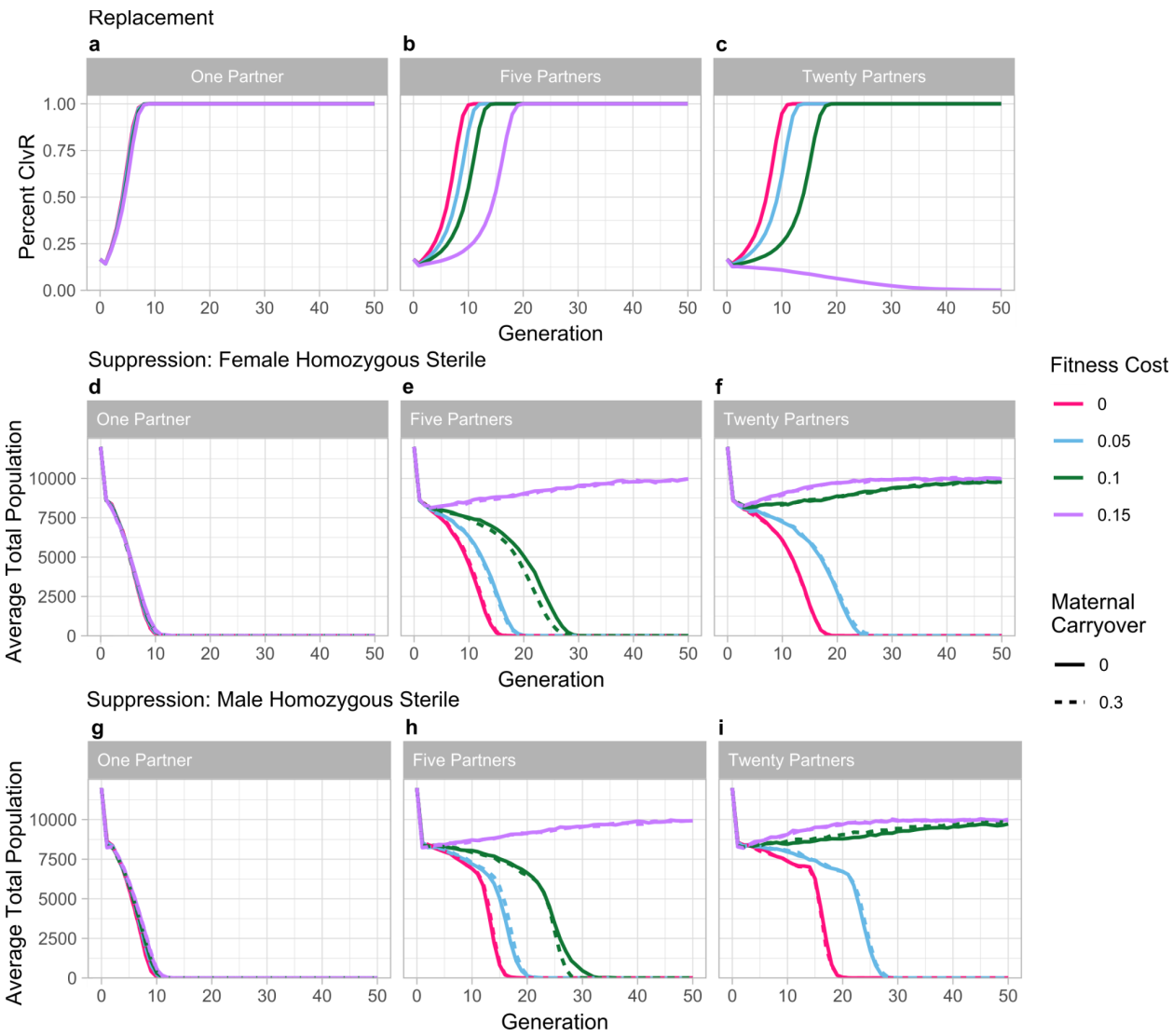
Extended Data Fig. 3: T3 heterozygous *ClvR* crosses for (a) female *ClvR*^{CaMV35S} and (b) male *ClvR*^{CaMV35S}. T3 *ClvR*^{CaMV35S}/+ heterozygotes were grown to adulthood and their ovules (left three columns) or pollen (right three columns) used in outcrosses to WT. Bar graphs show the number of siliques scored (red circles). The number of seeds within each silique scales with circle size. Counts are in Supplementary Table S3.



Extended Data Fig. 4: Images of individual (a) and whole pots (b) of heterozygous *ClvR*^{ubq}, homozygous *ClvR*^{ubq} and WT plants. In **a** individual plants have been removed from their pots and laid flat against a black background. **b** shows pots containing multiple plants.

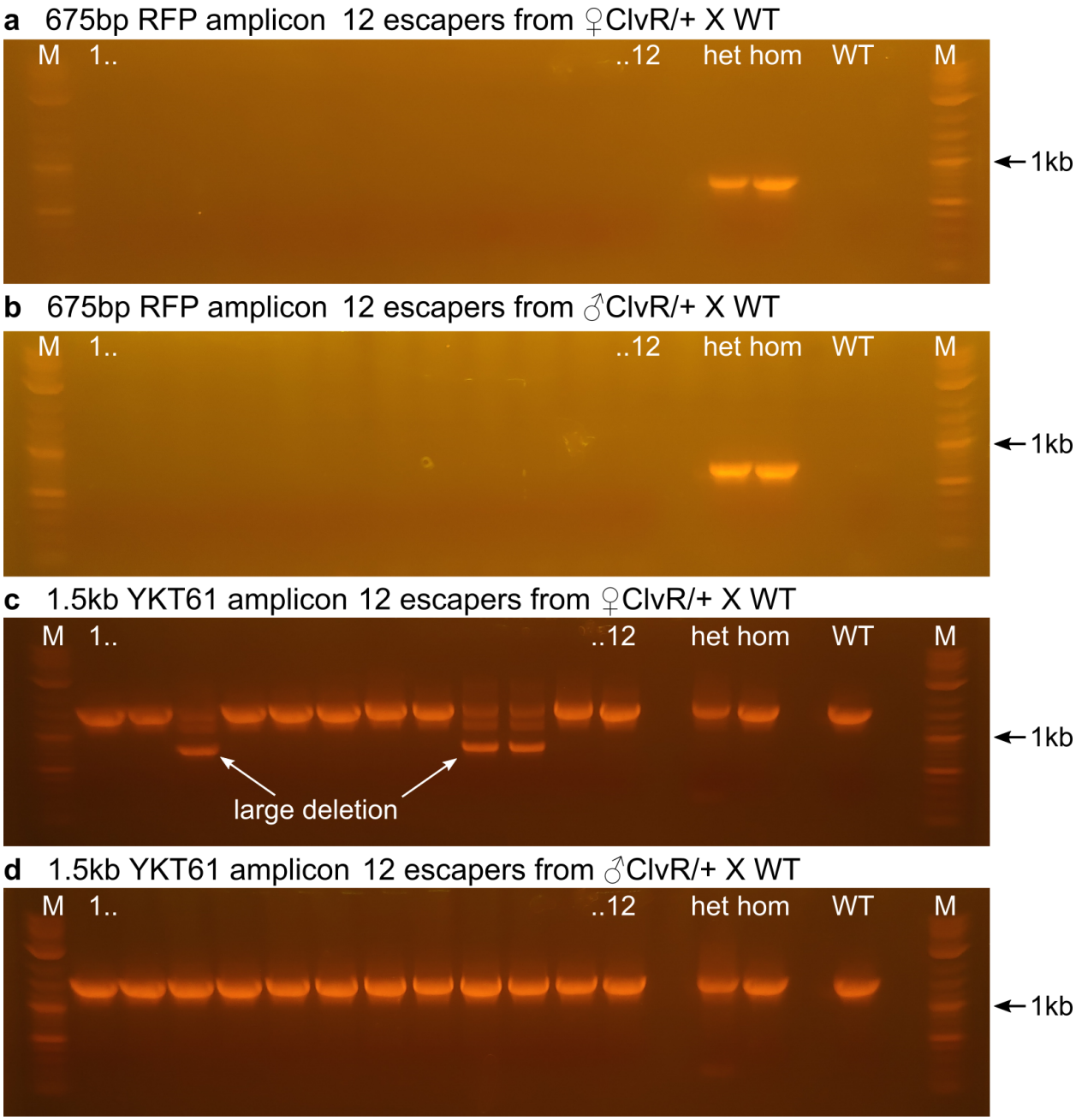


Extended Data Fig. 5: Crossing scheme for (A) T3 and (B) T4 crosses discussed in text and Fig. 2 and 3. (A) We selected 5 independent *ClvR*^{ubq} lines that showed 100% ClvR in the T2 self cross. Pollen of T2 plants was outcrossed to WT to generate T3 heterozyogtes. For each of these 5 independent lines we set up reciprocal crosses to WT with 4 plants per line (4 crosses/siliques per plant). **(B)** For 2 of the lines from (A) *ClvR*^{ubq6} and *ClvR*^{ubq7} we repeated the reciprocal crosses, with seeds coming from a ♀*ClvR*/+ or ♂*ClvR*/+ parent. For each of these we again crossed 4 plants (4 crosses/siliques per plant).

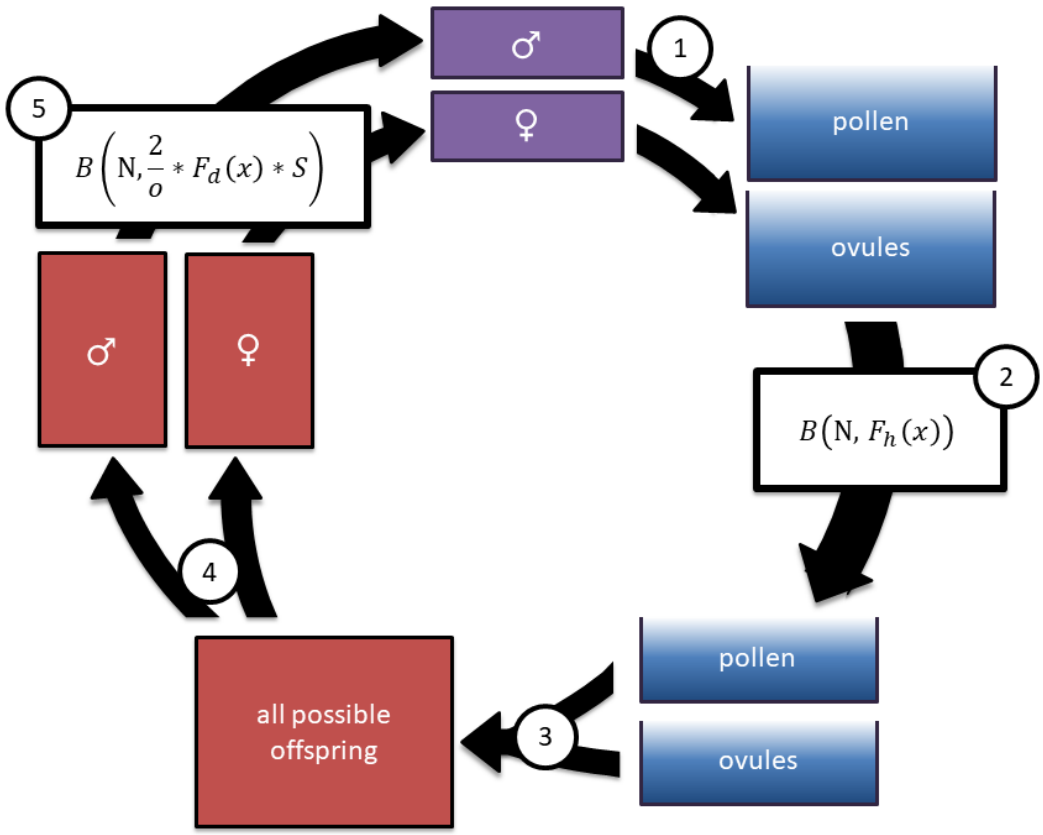


Extended Data Fig. 6: Predicted behavior of *ClvR* for population modification and suppression. (a-c) Population modification. *ClvR* is introduced as homozygous males at a frequency of 20% of the starting population, which is at carrying capacity, 10,000 individuals. The mating system is monogamous **(a)**, or polyandrous, with 5 males each providing 1/5th of the sperm needed to fertilize all ovules of an individual female **(b)**, or 20 males each providing 1/20th of the sperm needed **(c)**. Fitness costs are incurred by gametes (a probability of not being able to participate in fertilization, if chosen by the model). Maternal carryover is set to zero. Lines represent the average of 10 runs. **(d-f)** Population suppression with a transgene inserted into a recessive locus required for female sporophyte fertility. *ClvR* is introduced as above, at a frequency of 20%. The mating system is monogamous **(d)**, or polyandrous or polyandrous, with 5 males each providing 1/5th of the sperm needed to fertilize all ovules of an individual female **(e)**, or 20 males each providing 1/20th of the sperm needed **(f)**. Fitness costs are as above.

Maternal carryover is set to zero or 30% (the approximate value observed in our experiments with *ClvR*^{ubq}). **(g-i)**. As with **d-f**, but with the *ClvR* inserted into a locus required for male sporophyte fertility. For these simulations homozygous females were released into the population since homozygous males are sterile. Lines represent the average of 10 runs.



Extended Data Fig. 7: (a-b) PCR amplifications of a 675 bp DNA fragment of the RFP marker for escapers from a ♀ClvR/+ X WT **(a)** or ♂ClvR/+ X WT **(b)** cross. Hetero- and homozygous (het, hom) ClvR plants were used as positive controls, WT as negative control. Only ClvR-bearing plants showed the RFP band. **(c-d)** Control PCRs on the same DNA samples as in **a** and **b**, in which the YKT61 target region was amplified. Note some female escapers in **c** had larger deletions.



Extended Data Fig. 8: Modeling Process. Each generation starts with a pool of adult individuals, shown at the top in purple. This information is stored as a list where each index represents a possible genotype, and the associated value represents the number of individuals of that genotype. **(1)** For each mating, a single mother produces a pool of ovules and one (monogamous) or multiple (polygamous) males produce a single pool of pollen. **(2)** The number of ovules and pollen are reduced, based on their haploid fitness costs F_h such that a pollen with no fitness costs will have a 100% chance of survival, and a pollen with fitness cost 0.1 will only have a 90% chance of surviving. **(3)** From the pool of possible pollen and ovules, each ovule is mated with a single pollen to produce a possible offspring. **(4)** The possible offspring are randomly grouped as male or female **(5)**. The number of possible offspring is reduced to only those that survive. For each mating, the base chance of survival is 2 over the number of expected offspring *o*, as we expect each mating between a female and a male to produce 2 offspring when the population is at carrying capacity. This probability of survival is further modified by F_d, the fitness of the individual, and *S*. *S* denotes the density dependence function $S(P) = \frac{g}{1+(g-1)\frac{P}{K}}$ where P is the parent generation's population size, and K is the carrying capacity. *S* ranges from some growth factor $g = 6$ at low densities, to 1 at densities near carrying capacity. The offspring that survive become the parents of the

next generation.

Extended Data Table 1: Sequencing results of the YKT61 locus for various *ClvR* and escaper genotypes. “+” indicates insertions, “-” indicates deletions with numbers and type of bases deleted/inserted, WT indicates unaltered or wildtype sequences.

Heterozygous ClvR	gRNA1	gRNA2	gRNA3	gRNA4
1	-6TGCTCG	+1A	+1C	+1T
2	+1T / -6TGCTCG	+1A	+1C	+1T
3	+1T / -6TGCTCG	+1C	+1T	+1A
4	+1T	+1A	+1T	+1T
5	+1T / -1T	+1T	WT	+1T
6	-1T	+1T	+1T	WT
7	+1T	+1T / -1G	+1T	+1C
8	+1T	+1T	+1T	+1C
9	-95 / +1T	WT	+1T	+1A
10	+1T / -1T	+1C	+1T / -1T	-1C/+1A
inbred 1 generation	gRNA1	gRNA2	gRNA3	gRNA4
1	+1T	+1C	-1T	+1C
2	+1T	+1A	+1T	-1C
3	+1T	+1A	-1T	+1C
4	+1T	-1G / +1A	-1T	+1C
5	+1T / +1A	-1G	-1T	+1C
6	+1T	-5GTCAA	+1T	-1C
7	+1T / +1A	-1G	-1T	+1C
8	+1T	-2GC / +1A	-1T	+1C
Escapers from females	gRNA1	gRNA2	gRNA3	gRNA4
1a	-6TGCTCG	+1A	+1C	+1T
2a	-6TGCTCG	+1A	+1C	+1T
3a	+1T	WT	WT	WT
4a	+1T	WT	-2TC	WT
5a	WT	WT	+1C	+1T
6a	-6TGCTCG	+34	+1C	+1T
7a	WT	WT	WT	WT
8a	WT	WT	WT	-35
9a	+1T	WT	WT	+1C
10a	+1T	WT	WT	+1C

11a	-1T	WT	WT	WT
12a	-1T	WT	WT	WT
1b	+1T	+1T	WT	WT
2b	WT	WT	WT	WT
3b	+1T	WT	deletion between gRNA3 and 4	
4b	WT	WT	WT	WT
5b	+51bp indel	WT	WT	+1C
6b	+1T	WT	+1T	+1T
7b	+1T	WT	deletion between gRNA3 and 4	
8b	+1T	WT	deletion between gRNA3 and 4	
Escapers from males	gRNA1	gRNA2	gRNA3	gRNA4
1	WT	WT	WT	WT
2	WT	WT	WT	WT
3	WT	WT	WT	WT
4	WT	WT	WT	WT
5	WT	WT	WT	WT
6	WT	WT	WT	WT
7	WT	WT	WT	WT
8	WT	WT	WT	WT
9	WT	WT	WT	WT
10	WT	WT	WT	WT
11	WT	WT	WT	WT
12	WT	WT	WT	WT

Extended Data Table 2: Self crosses of escapers ♀*ClvR*/+ X ♂WT cross

T4 and T5 escapers from female *ClvR*/+			
escaper Plant	Seeds	aborted	% survival
1A	21	19	52.5
1B	21	21	50.0
1C	20	19	51.3
2A	22	24	47.8
2B	25	22	53.2
2C	23	21	52.3
3A	24	20	54.5
3B	23	22	51.1
3C	24	24	50.0
4A	26	23	53.1
4B	19	16	54.3
4C	23	21	52.3
5A	19	20	48.7
5B	16	20	44.4
5C	15	17	46.9
6A	21	20	51.2
6B	21	20	51.2
6C	19	20	48.7
7A	13	21	38.2
7B	16	16	50.0
7C	12	14	46.2
8A	19	19	50.0
8B	23	25	47.9
8C	17	20	45.9
9A	23	25	47.9
9B	27	20	57.4
9C	24	21	53.3
10A	23	21	52.3
10B	22	19	53.7
10C	25	21	54.3

11A	24	20	54.5
11B	22	26	45.8
11C	26	18	59.1
12A	12	24	33.3
12B	20	22	47.6
12C	18	21	46.2
Total	**748**	**742**	**50.2**
T5 escapers from male *ClvR*/+			
escaper Plant	Seeds	aborted	% survival
1A	50	1	98.0
1B	42	4	91.3
1C	50	0	100.0
2A	48	1	98.0
2B	46	1	97.9
2C	49	2	96.1
3A	51	0	100.0
3B	53	1	98.1
3C	49	1	98.0
4A	52	0	100.0
4B	50	1	98.0
4C	50	0	100.0
5A	40	1	97.6
5B	44	2	95.7
5C	41	1	97.6
Total	**715**	**16**	**97.8**
control, wildtype self cross: WT X WT			
Plant	Seeds	aborted	% survival
1	48	0	100.0
2	47	1	97.9
3	52	1	98.1
4	46	0	100.0
5	44	0	100.0

6	40	1	97.6
7	50	1	98.0
8	47	1	97.9
9	50	0	100.0
10	46	0	100.0
11	51	0	100.0
12	48	1	98.0
13	47	1	97.9
14	44	1	97.8
15	45	1	97.8
Total	**705**	**9**	**98.7**
homozygous *ClvR* self cross: *ClvR/ClvR X ClvR/ClvR*			
Plant	Seeds	aborted	% survival
1	49	1	98.0
2	56	0	100.0
3	54	0	100.0
4	41	2	95.3
5	41	0	100.0
6	42	0	100.0
7	52	0	100.0
8	50	1	98.0
9	47	1	97.9
10	48	0	100.0
11	54	1	98.2
12	49	1	98.0
13	41	2	95.3
14	40	2	95.2
15	40	1	97.6
Total	**704**	**12**	**98.3**