REVIEWER COMMENTS

Reviewer #4 (Remarks to the Author):

The authors largely address my comments, and I have no further question.
The reviewer #3 also have some comments on limited efficiency, lacking of certain experimental data and some inappropriate statements; and the authors gave their responses and arguments in this transferred manuscript.
Personally, I acknowledge the scientific novelty and importance of this study. However, the authors could better answer the questions of the reviewer #3 and could improve the presentation of the manuscript.
Specifically,
1. I understand the examination on mosaicism of F1 plants might be less reliable and hardly to achieve. Although "Super-Mendelian inheritance is not the main focus of the paper", a clear statement should be indicated to avoid misleading.
2. Obtain of homozygous F1 plants is the major merit of the study. As the reviewer #3 have pointed that, even in a proof-of-concept work, the low frequency may greatly restrict its potential application and compromise the significance. I think a perspective on a feasible future framework with essential details to improve efficiency of the selective inheritance system would be needed in the manuscript.

Reviewer #5 (Remarks to the Author):

I was asked to look at this manuscript as an additional reviewer, so I'll cut to the chase.
The type of gene drive here has been well tested and shown to work, but not yet in plants. It is based on cutting and gene conversion to copy the intact allele into the 'broken' allele. In the systems thus far tested, mostly in insects, this usually happens predominantly in the germline tissue, prior to gamete formation, and there is thus a bias of gametes containing the gene drive allele.

The authors do show evidence of copying and cutting, though unexpectedly (for me at least - though this may reflect my lack of knowledge about plants) this is not restricted to prior to or during gamete formation but is effectively a 'zygotic conversion'. This situation is quite different from insects, where the development of gene drives has had by far the most success to date. In some respects, the net result is similar, in that it can lead to biased inheritance regardless, as long as the germline in the zygote is also converted, though there are very different consequences for the utility of the drive - for example, in some scenarios (e.g suppression-type gene drives) it is useful, even essential, that 'homing'/copying occurs in the germline only, such that heterozygotes are carriers but do not show any other effects.
The rates of biased inheritance are very low for a gene drive, and a fair proportion of the copying events are imprecise, but I do accept that is a reasonable advance in the field of plants and there is much that can be learned for how to improve them to make them viable (that is not the same as 'proof of principle' though, and I would modulate the language accordingly).

It is not clear whether the a priori expectation was that zygotic conversion would be the predominant mode of bias - in insects (where this type of drive) is most developed it is not, so why here? Needs more context and rationale. Also, lines 38 and 39 are imprecise in describing prior art.
To me it is pretty clear that this article is written only with plant scientists in mind - terms and jargon need to be expanded or eliminated, respectively. The same is true for the title - "Selective inheritance of target genes from only one parent of sexually reproduced F1 progeny in Arabidopsis" will this appeal, or give any idea of the main findings or their significance, outside of a small subset of plant geneticists? Also, more background for the advantages of this type of technology - 'trait stacking' is mentioned. Sure I can get what this means but what are the current barriers to achieving it. Would the rates observed here solve the problem of trait stacking. Suppose there was 100% zygotic gene

conversion, how much would it help? Are there other uses for gene drive in plants? The article ends abruptly without any discussion of this.

As mentioned above, this situation here (zygotic conversion) is quite different from insects, where the development of gene drives has had by far the most success to date. In some respects, the net result is similar, in that it can lead to biased inheritance regardless, as long as the germline in the zygote is also converted, though there are very different consequences for the utility of the drive - for example, in some scenarios (e.g suppression-type gene drives) it is useful, even essential, that 'homing'/copying occurs in the germline only, such that heterozygotes are carriers but do not show any other effects.
What if it is not 'full' zygotic conversion - you might still get biased inheritance from a 'near' fully converted zygote but would it matter that the individual was not homogeneously homozygous?

It's not clear to me why you didn't cross heterozygotes to wild type. Even if there was no germline/meiotic gene conversion, and it was all zygotic, at least you would have had a much clearer signal - any homozygotes could only have arisen from zygotic gene conversion. This surely is much better than the self-pollination of the hets where you're looking for a deviation away from Mendelian expectation of 25%. This is why i find the T2 #128 homozygous crosses to WT more convincing, and the appearance of the homozygotes in the offspring that are suggestive of zygotic conversion, coupled with the (nice) haplotype analysis that shows mixed Col and Ler DNA that confirms it. - I realise you also did crosses (lines 207-216) of further homozygous drive x WT (though I almost missed it!) to back this last observation up. One question here, you never had the 'autonomous' gene drive on its own in any of your crosses, without the background source of Cas9? Did you try this? I would agree that the fact that you see an increase in conversion events with EC drive element over DMC drive element but I'd like to know why it wasn't done."

Rather than list all these points one by one I have included a copy of the annotated manuscript with my suggestions and queries.

Reviewer #4 (Remarks to the Author):

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Personally, I acknowledge the scientific novelty and importance of this study. However, the authors could better answer the questions of the reviewer #3 and could improve the presentation of the manuscript.

Response: Thank you for the positive comments.

Specifically,
1. I understand the examination on mosaicism of F1 plants might be less reliable and hardly to achieve.
Response: Thank you for understanding the experimental difficulties. We added the following sentence to line 196 to explicitly state the difficulties: "It is difficult to resolve the two possibilities with experimental approaches."

Although "Super-Mendelian inheritance is not the main focus of the paper", a clear statement should be indicated to avoid misleading.

Response: We have revised the text to remove the statements of "Super-Mendelian inheritance".

Line 55, the statement "We designed CRISPR/Cas9-based gene drives to test whether super-Mendelian inheritance can be achieved in *Arabidopsis* and whether heterozygous plants can be converted into homozygous plants (Fig. 1a, Extended Data Figs. 1, 2)" has been revised to "We designed CRISPR/Cas9-based gene drives to test whether such gene drives can perform "cutting and copying" of genetic elements in Arabidopsis (Fig. 1a, Extended Data Figs. 1, 2) and whether zygotic conversion can be achieved"

Line 394, "Figure 3. Super-Mendelian inheritance mediated by a CRISPR/Cas9-based gene drive." was changed to " Biased inheritance mediated by a CRISPR/Cas9-based gene drive"

2. Obtain of homozygous F1 plants is the major merit of the study. As the reviewer #3 have pointed that, even in a proof-of-concept work, the low frequency may greatly restrict its potential application and compromise the significance. I think a perspective on a feasible future framework with essential details to improve efficiency of the selective inheritance system would be needed in the manuscript.

Response: We would like to present our major findings without too much speculation. We added the following to the last paragraph, line 273.

"Identification of additional promoters that can drive Cas9 expression at a time that HDR is favored over NHEJ will likely also help improve the efficiency of gene drives in plants"

Reviewer #5 (Remarks to the Author):

I was asked to look at this manuscript as an additional reviewer, so I'll cut to the chase.

Response: We thank this reviewer for his/her effort in thoroughly reviewing this manuscript. The detailed and constructive comments from this reviewer are much appreciated by the authors.

The type of gene drive here has been well tested and shown to work, but not yet in plants. It is based on cutting and gene conversion to copy the intact allele into the 'broken' allele. In the systems thus far tested, mostly in insects, this usually happens predominantly in the germline tissue, prior to gamete formation, and there is thus a bias of gametes containing the gene drive allele.

Response: We agree with this reviewer that it is probably a good idea to explicitly differentiate the two different mechanisms: zygotic conversion and bias of gametes caused by gene drives. The first report of CRISPR/Cas9 gene drive (Gantz & Bier (2015) Science 348, 442-444) clearly demonstrated that zygotic conversions took place in Drosophila. It is true that other references we cited mainly dealt with germline biases. We revised the sentence in page 3, line 38-40, to the following:

"CRISPR/Cas9-based gene drives can convert a heterozygote into a homozygote in Drosophila by zygotic conversion [1] and enable super-Mendelian inheritance of a target gene in both mice and insects by generating a bias of preferred gametes [1-5]. Therefore, gene drives can potentially be used to force selective acquisition of target genes from only one parent in plants through zygotic conversion" (reference 1 is Gantz & Bier (2015) Science 348, 442-444)

The authors do show evidence of copying and cutting, though unexpectedly (for me at least - though this may reflect my lack of knowledge about plants) this is not restricted to prior to or during gamete formation but is effectively a 'zygotic conversion'. This situation is quite different from insects, where the development of gene drives has had by far the most success to date. In some respects, the net result is similar, in that it can lead to biased inheritance regardless, as long as the germline in the zygote is also converted, though there are very different consequences for the utility of the drive - for example, in some scenarios (e.g suppression-type gene drives) it is useful, even essential, that 'homing'/copying occurs in the germline only, such that heterozygotes are carriers but do not show any other effects.

Response: We agree with the reviewers' assessment of the utility of gene drives and the differences between plants and insects. We believe that showing successful cutting and copying in plants is a huge step forward in eventually using gene drives in plant breeding. We would like to point out that it will be a long way to gain any approval from government agencies to use gene drive for population suppression in plants. The applications of gene drives in plants in our opinion will be quite different from those in insects. The requirement of efficiency would be dramatically different. For example, 10% efficiency is probably not useful in population suppression, but it would be more than adequate in certain crop breeding applications.

The rates of biased inheritance are very low for a gene drive, and a fair proportion of the copying events are imprecise, but I do accept that is a reasonable advance in the field of plants

and there is much that can be learned for how to improve them to make them viable (that is not the same as 'proof of principle' though, and I would modulate the language accordingly).

Response: Thanks for the positive comment. We agree that the rate is low, but for certain applications, the current rate is adequate. For example, combining two traits that are conferred by two genes that are closely linked is almost impossible by traditional crosses. Using our gene drive, 1% to 2% F1 plants would have one homozygous and one heterozygous. Next generation, 25% of the plants would be double mutants. Compared to insects, the biased inheritance is indeed low.

We revised the last sentence of the abstract to "Our results demonstrate that homozygous F1 plants can be obtained through zygotic conversion using a CRISPR/Cas9-based gene drive"

It is not clear whether the a priori expectation was that zygotic conversion would be the predominant mode of bias - in insects (where this type of drive) is most developed it is not, so why here? Needs more context and rationale. Also, lines 38 and 39 are imprecise in describing prior art.

Response: Thank you for pointing out the imprecisions in line 38 and 39. As discussed above, although most studies in insects are involved in generating biased gametes, zygotic conversion was reported in Drosophila. We revised the sentenced to: "CRISPR/Cas9-based gene drives can convert a heterozygote into a homozygote in Drosophila by zygotic conversion [1] and enable super-Mendelian inheritance of a target gene in both mice and insects by generating a bias of preferred gametes [1-5]. Therefore, gene drives can potentially be used to force selective acquisition of target genes from only one parent in plants through zygotic conversion" (reference 1 is Gantz & Bier (2015) Science 348, 442-444)

The main goal of this study is to demonstrate that cutting and copying by HDR can be achieved in plants using CRISPR/Cas9. From the beginning, we expect that the efficiency would be much lower than those in insects (Please keep in mind that gene targeting by HDR has been notoriously difficult to achieve in plants). Zygotic conversion is the cleanest way to demonstrate "cutting and copying" by HDR because any positive homozygous F1 plants would provide strong evidence for "cutting and Copying.

To me it is pretty clear that this article is written only with plant scientists in mind - terms and jargon need to be expanded or eliminated, respectively. The same is true for the title - "Selective inheritance of target genes from only one parent of sexually reproduced F1 progeny in Arabidopsis" will this appeal, or give any idea of the main findings or their significance, outside of a small subset of plant geneticists? Also, more background for the advantages of this type of technology - 'trait stacking' is mentioned. Sure I can get what this means but what are the current barriers to achieving it. Would the rates observed here solve the problem of trait stacking. Suppose there was 100% zygotic gene conversion, how much would it help? Are there other uses for gene drive in plants? The article ends abruptly without any discussion of this.

Response: These are all excellent points. However, we are in a catch-22 situation. We were criticized by other reviewers for discussing potential applications (we had to remove those

languages in the version submitted to Nature Communications). We think that it is important to report the results first.

As mentioned above, this situation here (zygotic conversion) is quite different from insects, where the development of gene drives has had by far the most success to date. In some respects, the net result is similar, in that it can lead to biased inheritance regardless, as long as the germline in the zygote is also converted, though there are very different consequences for the utility of the drive - for example, in some scenarios (e.g suppression-type gene drives) it is useful, even essential, that 'homing'/copying occurs in the germline only, such that heterozygotes are carriers but do not show any other effects.
What if it is not 'full' zygotic conversion - you might still get biased inheritance from a 'near' fully converted zygote but would it matter that the individual was not homogeneously homozygous?

Response: We would like to emphasize again that potential applications of gene drives in plants are quite different from those in insects. Therefore, the consideration of efficiency would also be very different. The efficiency observed in this study might be considered far from adequate in insects. But for some applications such as combining two mutations from two plants, where the two genes are closely linked, the efficiency here is good enough.

Mosaicism is common in gene edited plants. We addressed this issue by analyzing the F2 plants of the apparent homozygous F1 plants. The F2 progeny tested were all homozygous, indicating 'full' zygotic conversion (Lines 167-168). From a practical point of view, even if F1 plants are mosaic, it is very easy to obtain stable, non-mosaic plants in the next generation.

It's not clear to me why you didn't cross heterozygotes to wild type. Even if there was no germline/meiotic gene conversion, and it was all zygotic, at least you would have had a much clearer signal - any homozygotes could only have arisen from zygotic gene conversion. This surely is much better than the self-pollination of the hets where you're looking for a deviation away from Mendelian expectation of 25%. This is why i find the T2 #128 homozygous crosses to WT more convincing, and the appearance of the homozygotes in the offspring that are suggestive of zygotic conversion, coupled with the (nice) haplotype analysis that shows mixed Col and Ler DNA that confirms it. - I realise you also did crosses (lines 207-216) of further homozygous drive x WT (though I almost missed it!) to back this last observation up.

Response: We assume that the reviewer is referring to the biased segregation in the F2 (line 175 to 195) and Figure 3. We agree with the reviewer that crossing a heterozygous to WT can provide a clean result about zygotic conversion, so does a cross between homozygous and WT, which we performed. Our results clearly demonstrated that zygotic conversion took place when we crossed homozygous plants to WT. The question is whether a cross between heterozygous and WT would definitively resolve the causes for the observed biased segregation in F2 plants? Any homozygous F1 would be indicative of zygotic conversion. A more than 50% of heterozygous F1 would suggest a germline/meiotic gene conversion. However, there is a caveat in such explanation. More than 50% heterozygous F1 could potentially be caused by chromosome killing during gametogenesis, not by gene drive activities per se. It is actually not trivial to differentiate the two possibilities.

It is useful to just report the observation without speculating too much about the actual mechanisms about the biased segregation here. We would like to stay with the main message, which is to obtain homozygous F1 plants by using gene drives.

One question here, you never had the 'autonomous' gene drive on its own in any of your crosses, without the background source of Cas9? Did you try this? I would agree that the fact that you see an increase in conversion events with EC drive element over DMC drive element but I'd like to know why it wasn't done."

Response: We agree with the reviewer that it would be very useful to get rid of the background Cas9. In plants, it is difficult to conduct gene targeting by HDR, which unfortunately is a prerequisite for constructing any CRISPR/Cas9-based gene drives. The breakthrough was reported in the *Nature Communications* paper three years ago (Miki, D., Zhang, W., Zeng, W., Feng, Z. & Zhu, J. K (2018). CRISPR/Cas9-mediated gene targeting in Arabidopsis using sequential transformation. *Nat Commun* **9**, 1967), which indicated that HDR needs a high level of Cas9 expression under the control of the Egg-cell specific promoter DD45. The Arabidopsis community has been using the Cas9-lines repoted in the *Nat Commun* paper. Unfortunately, the location and the nature of insertion of the Cas9 cassette were not known (we present the information in this manuscript), which makes genotyping difficult. We will certainly conduct such experiments in the near future.

Rather than list all these points one by one I have included a copy of the annotated manuscript with my suggestions and queries.

Response: Thank you for thoroughly evaluating the manuscript. We address the comments in the following paragraphs. The reviewer's comments are in quotation marks and in black. Our responses are in blue.

Comments directly on the manuscript:

Lines 23-24: "the only way this can happen is through zygotic conversion".
Yes, we agree that the homozygous plants in F1 could only be resulted from zygotic conversion.

Line 24: "but why not do CRY1/ cry1^{drive} X WT; that would be better surely or is there no way to score CRY1/ cry1^{drive} offspring easily?"

We agree that the heterozygous gene drive plants crossed to WT would have been a useful experiment to perform. But as discussed above, results can be subject to alternative interpretation. In addition, biased segregation in F2 is not a main focus of this study.

Lines 39-40: "this implies they are mechanistically different in the two organisms" "in germline cells" " this is largely not what is happening in the example cited-- rather, it is biased inheritance of one allele from one parent rather than acquisition of target genes from only one parent. I.e. they should receive another allele from the other parent"

The sentence has been revised to: "CRISPR/Cas9-based gene drives can convert a heterozygote into a homozygote in Drosophila by zygotic conversion [1] and enable super-Mendelian inheritance of a target gene in both mice and insects by generating a bias of preferred gametes [1-5]. Therefore, gene drives can potentially be used to force selective acquisition of target genes from only one parent in plants through zygotic conversion" (reference 1 is Gantz & Bier (2015) Science 348, 442-444)

Line 70-72: "recessive or dominant".

We revised the sentence to: because homozygous *cry1* mutants have long hypocotyls when grown in light

Lines 78: “Why? Two sources of Cas9”.

In order to achieve HDR-mediated gene targeting in Arabidopsis, it is necessary to have a high level of Cas9 in the system according to the literature (Two Cas9 alleles are used in accordance with a previous paper on HDR in *Arabidopsis* (Reference 8, Miki, D., Zhang, W., Zeng, W., Feng, Z. & Zhu, J. K. CRISPR/Cas9-mediated gene targeting in Arabidopsis using sequential transformation. Nat Commun 9, 1967, doi:10.1038/s41467-018-04416-0 (2018)). Our results actually confirmed their findings as well.

Line 70-72: “If it needs to be 'released' presumably this is only from the transforming plasmid i.e. gRNA 2 [unclear] plasmid sequence [aside] the homology arms? This is usually not necessary in many organisms. If it is a plant thing, it is worth [stating unclear]”

Many aspects of HDR in plants are still mysterious. In literature, some people do not release the repair template from plasmids while others do. It is not clear whether it is necessary or not, given that nobody achieved efficient HDR through agrobacterium-mediated transformation until the report in the above mentioned *Nature Communication* paper.

We use the second gRNA to release the gene drive unit in the hopes that removing DNA sequence distal to the left and right homology arms will increase the chances of precise HDR editing. At least we thought that it would not hurt. Another consideration is that plasmids from Agrobacteria will integrate in the genome randomly. We reasoned that by incorporating a gRNA-mediated releasing mechanism, the template would still be available even if the plasmid is integrated first.

Line 86: “syntax -- it is not 4.68% of HDR events but 4.68% of all events”

We have fixed the syntax of this line. It now reads: “We observed that 4.68% of the T1 transformants had the gene drive fragment precisely placed in the *CRY1* gene via HDR (Table 1).

Line 93: “presumably this was already done when calculating “complete HDR” events versus left HDR events, right HDR events etc.? So why is it now written as sequential to Table 1 - did this not use P1/P2 and P3/P4 (in Fig 1A)”

Sorry for the confusion. The primers P1/P2 and P3/P4 can only check for HDR events at the borders of the homology arms. Keep in mind that the gene drive element is larger than 10 kb. The long PCR reactions were necessary to make sure that the entire donor element was inserted.

Line 98: “original plasmid”.

We changed wording to “the gene drive element in the original plasmid would not be amplified”

Line 100: “were HDR lines other than #128 checked this way? were they also precise? - I ask because later, apparent precise events were not so (e.g line 151)”

The other lines were also checked in the same way. We added the following sentence to line 209: Our long PCR reactions similar to that shown in Extended data Fig. 4 confirmed that the complete gene drive elements were present in the two lines

Line 107: “element”.
We have changed instances of “donor fragment” to “donor element.”
We have also changed “fragment” to “element” where it appears appropriate.

Line 108: “Is this the correct term? You mean it has numerous defunct DNA polymorphisms that allow [doubles] of its DNA from [this or the other”

Yes, we think polymorphic is the correct term. Ler and Col have many polymorphic regions, which have been widely used for designing DNA markers.

Lines 109-111: “so your expectation was to see zygotic conversion, rather than pre-meiotic or meiotic gene conversion? That is not what would be expected from other systems so why are you ‘expecting’ it here?”

This particular experiment can only detect zygotic conversion and cannot detect pre-meiotic or meiotic gene conversion because we cross the homozygous line to Ler.

Lines 140-141: “If you did it and [looks] like you did, why not tell us the extent of the gene conversion event on the left arm if you could resolve it with the sequencing?

We could not resolve the left arm junction by PCR and Sanger sequencing. We will need to conduct whole genome sequencing to resolve this. We have sequenced (whole genome sequencing) some other gene targeting lines (not gene drive lines) that inserted GFP in the target genes (such as the NPY5 line presented in this paper). What we found is that some lines have one side perfectly inserted through HDR, but the other side has very complex patterns such as several copies of plasmid DNA inserted. It is not trivial to resolve the imperfect junctions by simple PCR/Sanger sequencing.

Line 149 “Consistent with RA marker [reaction] analysis (line 135 and figure 2B).

Yes

Lines 153-165: “So these events would have been called 'complete' HDR events -- Table 1 but 3 of 5 of them [impure]?

If the PCR primers used for the T1 plants in Table 1 were used here, we would have discovered the imprecise/one-sided HDR events earlier. We used different primers to determine

polymorphic difference in F1 simply because it was easier to resolve the differences in gels. We have clearly described what happened.

Line 149 “if the same imprecision rates applied there, as here”.
the sample size of the F1 plants is too small to make such a comparison.

Line 166: “Crossed to WT or homozygous cry1”.

We changed the wording to: we analyzed at least 48 F2 progeny resulting from self-pollination of each identified homozygous F1 plant.

Line 170: “Why would it not be? Were the F1 parents in line egg female then?

The handwriting was difficult to interpret here. Although Cas9 is expressed under the egg cell specific promoters and is presumably only expressed in egg cells, we thought it is prudent to test whether or not conversion would occur when the parent contributing the gene drive element was male.

Line 171: “homozygous”
“Homozygous” added

Line 173: “it did not function in zygotic conversion - the only way it could so is if there was extensive paternal deposition. But did it show any drive in the germline - i.e. if you cross males heterozygous for the gene drive to WT females do you get biased inheritance?”

Whether there is any drive in the germline in our system has not been resolved. The cross between heterozygous and WT is an excellent suggestion, but it can lead to convincing results only if meiotic conversion is efficient enough. If the rate is similar to what we have observed for zygotic conversion, the results would be ambiguous.

Line 175 “This is the definition of a gene drive-- if it doesn't do this, it's not a gene drive”.

We agree. That is why we conducted such experiments.

Lines 180-181: “You mean that the overabundance of cry1 / cry1 is made up by a deficit in cry1 / cry1 and not a deficit in WT? It would be cleaner to state it as such”.
We agree. We changed the sentences to: Interestingly, the number of wild type plants (*CRY1/CRY1*) in the F2 population was close to what is expected from Mendelian segregation (22.4% (74) vs 25% expected). Our results suggested that the observed additional homozygous *cry1/cry1* plants were mainly generated by converting heterozygous (*CRY1/cry1*) plants into homozygous plants by the gene drive ($p = 0.28$, One-Sample Binomial Test) (Fig. 3).

Line 191: “This needs an asterisk on the figure legend for the 22.4% that were denoted as cry1 / cry1”.

Thank you for spotting this. We have revised both the figure and the legend to reflect this. The legend now reads: “**Figure 3. Biased inheritance mediated by a CRISPR/Cas9-based gene drive.** A plant with a wild type (WT) *CRY1* copy in one chromosome and the gene drive element (Red) inserted in *CRY1* in the homologous chromosome underwent self-pollination to produce progeny. Several genotypes are expected: both copies of *CRY1* are WT (*CRY1/CRY1*); one copy of *CRY1* had NHEJ without disrupting *CRY1* function such as a 3 bp deletion (*cry1*‡); one copy of *CRY1* had NHEJ resulting in loss of *CRY1* function (*cry1**); heterozygous *cry1*; homozygous *cry1*; and plants with one copy of the gene drive insertion and one copy of *cry1* that had undergone NHEJ repair (*cry1/cry1**) that disrupted *CRY1* function. The genotypes of *CRY1/CRY1*, *CRY1/cry1*‡, *CRY1/cry1**, *CRY1/cry1, and cry1/cry1*‡ were not phenotypically different. Plants with either *cry1/cry1* or *cry1/cry1** genotypes display long hypocotyls in light conditions.”

We revised line 190 to 192 to the following: “Note that the apparent *CRY1/CRY1* plants might also contain NHEJ events that did or did not result in a frameshift (*CRY1/cry1*‡ and *CRY1/cry1**). The small number of (*cry1/cry1*‡) might also contain NHEJ events such as three base pair deletions (*cry1/cry1*‡) that did not result in loss of function. Therefore, the number of NHEJ events presented above was likely underestimated.”

Line 195:” So T2 is a distinct terminology, different from F2? Is this plant jargon? "Transgenic generation 2"?

Yes, this is plant biology jargon. T2 plants are the direct offspring of T1 plants as a result of self-pollination. Because this jargon has been widely used in plant biology literature, we think that it is fine to leave as is.

Line 197: “I probably agree with this interpretation but why not do the more definitive test of crossing a heterozygous gene drive individual and crossing to WT to look for biased inheritance? or maybe cutting events do happen during or prior to meiosis but these are the NHEJ events and the HDR events can only occur in the early zygote?”

Please see above about our explanations above about the heterozygous cross.

Lines 197-198: “2 / 192? so no germline specific [unclear] in parents, and 2 / 192 events result from zygote [unclear].

Part of the handwriting was not clear. If we understand the comment correctly, our results indicate 2 zygotic conversions (this experiment cannot detect meiotic conversion)

Line 197 “so two events out of 192, versus zero events out of 192”.

Yes, that is correct.

Line 205:"early zygotic"
We have changed this line to make it clearer. It now reads: "Our results suggest that the presence of Cas9 in the egg cell might facilitate successful early zygotic HDR in *Arabidopsis*."

Line 223: "this statement is misleading, in that it implies the achievement of a useful gene drive is a fait accompli, when it is far from it (judging by the results presented herein), and that the only issue with it is it being classified as 'transgenic'.

Our original statement is probably too strong/inaccurate. The message we want to convey is that governments probably would not approve any commercialization of crops that still harbor Cas9. Anyway, we revised the sentence to:

Presence of canonical gene drives in crops will likely set off alarms to the public and government regulatory agencies because of the concerns surrounding the presence of transgenes such as selection markers and *Cas9*.

Line 249: "Table"
This is just one piece of data and we do not think that a table is necessary.

Lines 256-257: "Crossed to homozygous?

Not crossed, just self-pollination. We have added the clarifying text to the end of the line. It now reads: "We also genotyped the F2 progeny from the three homozygous plants and the F2 plants were all homozygous, consistent with an early HDR event or full zygotic conversion, rather than mosaicism."

Line 330: "Can this nomenclature not be changed? While it is explained in the legend, it is still confusing to have two different names for the same promoter driver [unclear]".

We know that this can be very confusing. But the two promoters were actually different. One is a hybrid of EC1.1 and EC1.2 while DD45 is just EC1.2. Lines 54 and 76-77 differentiate them.

Figure 2: "Why switch these rows? It's confusing. But on panel b it looks like the bs11 row is on the col DNA (black)"

Thank you for pointing this out, we have switched the chromosomes around in figure 2b.

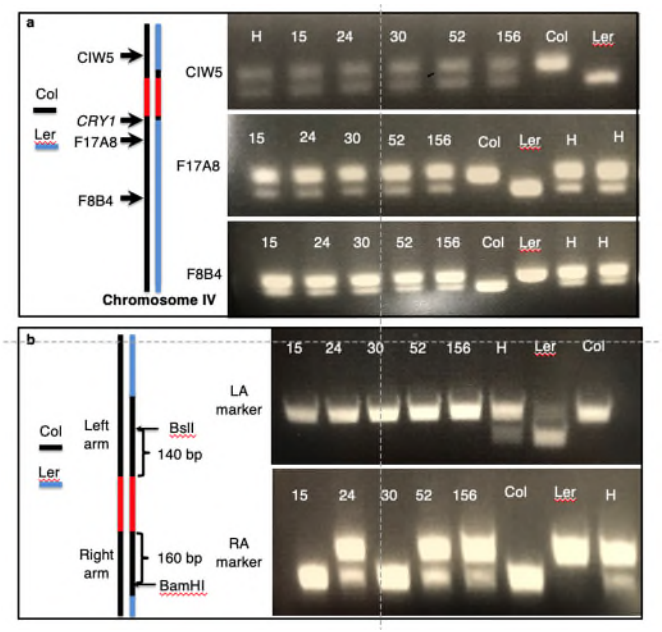

Figure 3: Would be nice and cleaner to state this early in the main text e.g. line 72

The re-worded line 72 should clear this up now.

Figure 4: “Mosaic or wholly converted zygotes? “
These are wholly converted zygotes to the best of our knowledge. We analyzed F2 plants and found them all to be homozygous.

REVIEWERS' COMMENTS

Reviewer #5 (Remarks to the Author):

the responses to reviewers' comments are quite thorough, and appreciated.
There are just two outstanding items:
the confusion in the terminology is coming from the fact that though one of the elements could be described as a canonical, autonomous, gene drive element in its design, it has not been demonstrated to act as such, in the same way that you might build a canonical car with chassis, engine, wheels etc. but if you only show it moving when pulled by a tow truck then you can't say it is working, canonically, as a car. It puzzles me why one should entertain possible confusion or misunderstanding on this when, as the authors point out, that is not the focus of the paper and its selling point is more about progressing the workability of HDR approaches and potential applications in breeding.
Following on from that, and I do sympathise with the author's catch-22 comment, surely some of the points, or a briefer version of them, relevant to how much this could help in crop breeding would really help the impact and broader readability of the text - they are well made in the response to reviewers. Consider this a plea to the Editor to allow an improvement to the article.

Lastly, the statement that was changed to "Presence of canonical gene drives in crops will likely set off alarms to the public and
government regulatory agencies because of the concerns surrounding the presence of transgenes such as selection markers and Cas9" still leaves ambiguity - as written they should set off no more concern than any transgenic plant, or any non-canonical gene drive (since this would also have transgenes as Cas9). So why do they set off concerns - because an autonomous gene drive showing high rates of biased inheritance would be highly invasive , but that was not shown here. While I fully accept that improvements, building on the work herein, might get us to that point I think you will appreciate that this statement needs more nuance and subtlety.

I do not need to see the manuscript again, these are issues that can and should be resolved editorially. I remain of the opinion that this will be a valuable article for the field.

REVIEWERS' COMMENTS

Reviewer #5 (Remarks to the Author):

the responses to reviewers' comments are quite thorough, and appreciated.

Response: Thank you for the positive comments.

There are just two outstanding items:

the confusion in the terminology is coming from the fact that though one of the elements could be described as a canonical, autonomous, gene drive element *in its design*, it has not been demonstrated to act as such, in the same way that you might build a canonical car with chassis, engine, wheels etc. but if you only show it moving when pulled by a tow truck then you can't say it is working, canonically, as a car. It puzzles me why one should entertain possible confusion or misunderstanding on this when, as the authors point out, that is not the focus of the paper and its selling point is more about progressing the workability of HDR approaches and potential applications in breeding.

Response: Our understanding of a “canonical gene drive” is that the entire gene drive unit (Cas9, gRNA elements and cargo) is responsible for both the cutting and being copied. In that sense, we have clearly demonstrated the cutting and copying, as we identified homozygous F1 plants. It is true that the efficiency is much lower than that observed in insects. The comparison to building a car is not very accurate here because the cutting and copying we observed was really driven by the gene drive elements. Because the term is defined the in the text, readers will not be confused.

We appreciate that the reviewer taught us the nuances of gene drive terminology.

Following on from that, and I do sympathise with the author's catch-22 comment, surely some of the points, or a briefer version of them, relevant to how much this could help in crop breeding would really help the impact and broader readability of the text - they are well made in the response to reviewers. Consider this a plea to the Editor to allow an improvement to the article.

We appreciate that this reviewer is advocating on our behalf. We would like to stick to the results without extrapolating too much. In the end, making the discoveries

public is more important.

Lastly, the statement that was changed to "Presence of canonical gene drives in crops will likely set off alarms to the public and government regulatory agencies because of the concerns surrounding the presence of transgenes such as selection markers and Cas9" still leaves ambiguity - as written they should set off no more concern than any transgenic plant, or any non-canonical gene drive (since this would also have transgenes as Cas9). So why do they set off concerns - because an autonomous gene drive showing high rates of biased inheritance would be highly invasive , but that was not shown here. While I fully accept that improvements, building on the work herein, might get us to that point I think you will appreciate that this statement needs more nuance and subtlety.

Response: We agree with the reviewer. We revised the text to avoid judgement on the impact of public opinion or regulatory policies. The new text simply states the fact: “However, a canonical gene drive and its target gene are located in the same locus, making them inseparable”

I do not need to see the manuscript again, these are issues that can and should be resolved editorially. I remain of the opinion that this will be a valuable article for the field.

Response: Thank you for your inputs and the positive comments.