APETALA2 functions as a temporal factor together with BLADE-ON-PETIOLE2 and MADS29 to control flower and grain development in barley

Jennifer R. Shoesmith, Charles Ugochukwu Solomon, Xiujuan Yang, Laura G. Wilkinson, Scott Sheldrick, Ewan van Eijden, Sanne Couwenberg, Laura M. Pugh, Mhmoud Eskan, Jennifer Stephens, Abdellah Barakate, Sinéad Drea, Kelly Houston, Matthew R. Tucker and Sarah M. McKim
DOI: 10.1242/dev.194894

Editor: Ykä Helariutta

Review timeline
Original submission: 21 July 2020
Editorial decision: 1 September 2020
First revision received: 30 December 2020
Accepted: 25 January 2021

Original submission

First decision letter

MS ID#: DEVELOP/2020/194894

MS TITLE: APETALA2 functions together with BLADE-ON-PETIOLE2 and MADS29 as a temporal factor to control flower and grain development in barley

AUTHORS: Jennifer R Shoesmith, Charles Ugochukwu Solomon, Xiujuan Yang, Laura G Wilkinson, Scott Sheldrick, Ewan van Eijden, Sanne Couwenberg, Jennifer Stephens, Abdellah Barakate, Sinead Drea, Kelly Houston, Matthew R Tucker, and Sarah M McKim

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

In this work Shoesmith and colleagues characterized loss of function mutations in the Barley gene HvAP2, which has important roles in the regulation of plant height, spike architecture and lodicule development. By studying different hvap2 mutants, the authors revealed the role of HvAP2 in the control floret organ identity and grain development. Also, by reanalyzing a gain-of-function mutant (Zeo1, previously described by this group), the authors confirmed published results on the role of AP2-like genes in the promotion of floret fate in cereals.
Interestingly the author showed that HvAP2 is required for maternal tissue differentiation and elimination during grain development. The authors provided evidence that HvAP2 may control grain development through the regulation of MADS29 expression, whose inactivation severally affect grain development.
Moreover, the authors studied the interaction between HvAP2 and LAXATUM.A/BLADE-ON-PETIOLE2 (HvBOP2). The results indicated that HvAP2 and HvBOP2 interact to control spike density and lodicule identity.
I think the work is very well organized and presents interesting observations.
I have some comments and suggestions for the authors to consider.

Comments for the author

Major comments

>About the HvAP2 functions during early floral development The authors state: “These data support that HvAP2 promotes HvMADS1 expression, potentially explaining the loss of perianth features in gigas1.a and the glume to lemma homeotic transformations in Zeo1.b (lines 206-208)”.
The authors show higher MADS1 expression in Zeo1.b by qPCR and in situ however no change in MADS1 expression is shown in gigas1.a. Therefore, the role of HvMASD1 in the loss of perianth features in gigas1.a is not clear. I understand that the floret organ phenotypes in hvap2 mutants are subtle and it could be hard to see differences in MADS gene expression by qPCR. Did the authors attempt to do MADS1 in situ in Hvap2 mutants?
In addition, it is not very clear how RT-qPCR data was normalized and expressed in the graphs. However, there seems to be an increase in MADS1 expression levels from WD3.5 to WD4 in all genotypes (Figure S7). That may indicate that MADS1 expression pattern is controlled independently of HvAP2 which instead may modulate quantitatively MADS1 expression level.

>HvAP2 in grain development.
The authors describe the role of HvAP2 in maternal tissue differentiation and elimination during grain development. They suggest JA and MADS29 as putative mediators of HvAP2 functions.
Have the authors tested the effect exogenous JA on grain development in hvap2-2 or gigas1.a mutants?
The connection between HvAP2 and MADS29 is very interesting, however the evidence for this interaction is mainly a qPCR result showing a reduced MADS29 expression in hvap2 mutant (gigas1.a). The authors indicated that hvap2 and hvmads29 mutants have similar grain phenotypes, however without results showing a genetic interaction it is difficult to conclude about a connection between these two genes. A cross between Zeo1.b and hvmads29 would be informative.
Have the authors check MADS29 expression in hvap2 mutants by in situ?

A point not explored by the authors is how HvAP2 controls grain length. It could be interesting to check for changes in cell size and cell number. The shorter grains of Zeo1.b reminds the phenotype of transgenic Arabidopsis expressing an AP2 version that is immune to miR172 and MIM172 constructs described in Ripoll et al., 2015 “microRNA regulation of fruit growth”.

>HvAP2 acts downstream of HvBOP2 to control lodicule identity and grain length.
The authors provide genetic interaction data between HvAP2 and HvBOP2 in the control of lodicule identity and spike density. However, I do not see a clear interaction in grain length. Moreover, the authors description of the grain length phenotype appears contradictory:
Line 348 "Zeo1.b lax.a grain length was equivalent to lax.a (p=0.26; Fig. S14)".

Lines 354-355 "Zeo1.b lax.a double mutants also showed striking epistasis in specific features. Double mutants showed Zeo1.b-like spike density ($p \leq 0.001$), grain length ($p < 0.05$)"
Could the authors clarify this?

The interaction HvAP2 and HvBOP2 in lodicule identity is very interesting.
The authors also mentioned that "3% of hvap2-2 florets green, filament-like organs with smooth and hairy bract-like regions replaced the lodicules (Fig. 2G, H)". To me, those filament-like organs looks like stamens. Have the authors check for filament-like organs (or stamens) in lodicules of gigas1.a lax.a/+ plants?

Additionally, it is not very clear to me whether HvBOP2 acts upstream or HvBOP2 and HvAP2 act parallelly to control lodicule identity? To clarify, I think it is important to check HvAP2 expression in lax.a mutants.

Finally, I have a comment about the regulation of grain and hull length. The authors discuss that HvAP2 may modulate grain length by influencing hull length (line 443). However, it seems that Zeo1.b lax.a double mutant has longer lemmas (Fig 6F) but shorter grains (Fig 6J) than the controls. Therefore, based on the authors results elongation of these two organs could actually be uncoupled. Could the author comment on this?

Minor comments
Line 125 "which were decorated with ectopic glume-like hairs". Are these, glume specific or more general bract-like hairs?
Line 130 Remove Fig. S2. Fig1 O is not referred.
Line 132 "although gigas1.a lemma width was unchanged compared to Bowman (Fig. S2)". This is not shown in figure S2. However, Figure S4A shows significant wider lemmas in gigas1.a Line 138 I suggest including lax spikes phenotype in the description
Line 144. BOPA1 or BOPA2?
Line 159 "HvCas9". I understand that the authors mean a barley codon optimized Cas9. However, the nomenclature HvCas9 seems not appropriated.
Line 162 "deletion (175bp–240bp)". Should not it be (175bp to 214bp)?
Line 176 What do the authors mean by perianth boundary? Lemma-awn boundary?
Line 213 MADS3 and MADS58. The same barley locus number (HORVU3Hr1G026650.1) is indicated for both genes. Besides, if authors refer to orthologs of Rice MADS3 and MADS58, both are C-class AGAMOUS-like genes (Yamaguchi et al., Plant Cell 2006).
Line 304 I could not see expression in peripheral vascular bundles. Could you please indicate in the figure?
Line 341 "($p < 0.04$; Fig. 6C–D; Fig. S14)" Should not it be Fig S13?
Line 347-348 "S14 and 15". Should not it be FigS13 and S14 instead?
Line 437 "(Fig 1D–F;..)". Should not it be Fig 1D, O?
Line 461 "Fig5G" should be Fig4H.

Figure 1N I suggest indicating the position of glumes and lemma-like glumes.
Figure 2F Is the scale for GP and hvap2-2 the same? It looks like hvap2-2 is a little bigger.
Figure 3C I suggest replacing WD7 by WD9. I cannot see very clear differences between lodicules at WD7.
Figure 3C and E. Could you indicate how many replicates were performed?
Figure 4C-G. Could you indicate how many replicates were performed?
Figure 5B. Could you indicate how many replicates were performed?
Figure S8 It is labeled as FigS7
Figure S10B What is the purpose of MADS1 sense in situ?
Figure S2 and S14. Some discrepancies in Zeo1.b grain width parameters.

Methods
Could you please clarify how RT-qPCR data was normalized? Which gene was used as control gene? How are expression levels express?

Reviewer 2

Advance summary and potential significance to field

This manuscript deals with the functional characterization of HvAP2 in barley.
In a previous paper the authors describe the role of HvAP2 in internode regulation. In this manuscript they focused on the role in floret organ determination and grain development and compared it with described functions from orthologous genes from rice and wheat. Several functions of this pleiotropic gene appeared to be conserved, but also new roles in ovule and grain development are reported. A novel part of the work is that part of the network of HvAP2 is elucidated, i.e. an important downstream and a putative upstream regulator have been found and are involved in some of the functions of HvAP2. The results are solid and well described in the figures.

Detailed description of the work:
Searching for mutants of HvAP2, knowing the phenotype of the gain-of function mutant Zeo1.b brought the authors to an 'old' mutant (reported in 1962) that overlapped with the HvAP2 locus. This mutant, gigas1.a showed multiple elongated features in the spike and spikelets. Shape of spikelets and grains were more lance-shaped, and awns at the tip of lemmas and glumes affected suggesting more glume awn-like characteristics of the lemma awn in the mutant.
Lodicules develop ectopic bract-like lamina, decorated with ectopic glume-like hairs. In the gain-of HvAP2 mutant Zeo1.b the glumes were often transformed into lemmas, similar to miR172 resistant versions of TaAP2L in wheat and in line with a lemma identity function for HvAP2. Besides these partial homeotic transformations that mimic a typical A-type mutant, other aspects of female organ and grain development were affected.
Genomic analysis of the gigas1.a mutant revealed that it has a deletion comprising HvAP2 and six other genes. To confirm that the phenotype of the mutant is caused by the deletion of HvAP2, CRISPR/Cas9 mutants were generated and one CRISPR deletion mutant largely phenocopied gigas1.a. Based on these mutants, the authors propose that HvAP2 defines the identity of the outer perianth organs, increases stigmatic papillae branching and widens grain while also restricting longitudinal growth of spike internodes, spikelets floret organs and grain.
Searching for MADS box members that could act downstream of HvVAP2 in the florets, revealed HvMADS1, orthologous to the rice LHS1 gene, as a candidate.
These two barley genes show an overlap in gene expression and the lhs1 mutant phenotype hints towards a similarity with gigas1.a. HvMADS1 is higher expressed in Zeo1.b, but not affected in gigas1.a (fig 3). This latter is not mentioned in the text but is relevant, because it doesn't fit with the conclusion that the loss of perianth features and homeotic transformations in gigas1.a can be explained by a reduction of HvMADS1 (line 206-208).
Other MADS-box genes, belonging to the C and D-class could potentially account for the gigas1.a phenotypes in the pistil, lodicule and stigma. This is based on expression of the MADS box genes in the loss and gain-of function mutants.
A detailed phenotypic analysis of WT, Zeo1.b and gigas1.a, including histology (fig 4) was performed showing defects in the nucellus and integument layers of the developing seed. These analysis suggested that HvAP2 promotes degradation of maternal tissues during seed development.
A potentially downstream acting MADS box gene in the grain is HvMADS29, which is orthologous to OsMADS29. Downregulation of OsMADS29 in rice inhibits degradation of maternal tissues, including nucellus, leading to shrunken seeds. HvMADS29 was downregulated in the gigas1.a mutant, suggesting that it is controlled (directly or indirectly) by HvAP2. The overlap in expression of HvMADS29 and HvAP2 is in developing ovules, in particular in the vascular bundles, but while the expression of HvAP2 goes down during grain development HvMADS29 expression is maintained, indicating not a complete epistasis. CRISPR derived hvmads29 mutants showed a number of defects similar to gigas1.a suggesting that HvAP2 acts upstream of HvMADS29 and is involved in the formation of vascular tissue in the nucellus.
Based on the previous work of the authors (2019) they investigated whether JA is also involved in barley grain development. Two JA-responsive genes JIP23 and JIP60 are downregulated in the gigas1.a mutant.
To search for upstream regulators of HvAP2, the authors found an existing mutant (lax.a) with a deleted HvBOP2 transcription factor gene and displaying multiple defects that phenocopied gigas1.a. The double mutant lax.a Zeo1.b revealed also independent characteristics, indicating a partial epistasis.

This suggests a genetic interaction between HvBOP2 and HvAP2, but I am missing expression studies of HvAP2 in the lax.a mutant background, to understand the transcriptional relationship between the transcription factor and the putative downstream target. Since HvAP2 can be post-transcriptionally regulated by HvmiR172, it might be informative to test the expression of the MiRNA genes as well.

Comments for the author

I have two suggestions for improvement:

1. I am missing expression studies of HvAP2 in the lax.a mutant background, to understand the transcriptional relationship between the transcription factor and the putative downstream target. Since HvAP2 can be post-transcriptionally regulated by HvmiR172, it might be informative to test the expression of the MiRNA genes as well.

2. This manuscript nicely shows some putative downstream genes and a putative upstream transcription factor of the pleiotropic gene HvAP2. Some of the functions are new, but a number has been revealed from orthologous genes in rice, wheat or even Arabidopsis. I would like to see a concluding figure with the different MADS genes, HvBOP2 and possibly other known factors that illustrates the HvAP2 network and its pleiotropic functions.

Reviewer 3

Advance summary and potential significance to field

The manuscript by Shoesmith et al. describes the function of HvAP2 controlling floret organ identity, floret boundaries and maternal tissue differentiation and elimination during grain development in Barley. The authors characterize the HvAP2 function by loss-of-function mutants generated by CRISPR-Cas9. Furthermore, expression analyses are performed to observe relationships with MADS domain proteins of the ABCDE model. A detailed analysis is performed on the HvMADS29 protein, a B-sister MADS protein, again using CRISPR loss-of-function alleles were generated and phenotypically analyzed, some overlap in phenotypes is observed compared to the Hvap2 mutants. Finally, generating double mutants, the authors show that HvAP2 functions downstream of HvBOP2.
This story provides nice functional and molecular insight in floret and grain development in Barley. The authors are experts in the theme. All experiments are of high quality and presented in good figures. Writing is excellent.
I do not have any comment or suggestion to improve this manuscript.

Comments for the author

The authors are experts in the theme. All experiments are of high quality and presented in good figures. Writing is excellent.
I do not have any comment or suggestion to improve this manuscript.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field...

In this work Shoesmith and colleagues characterized loss of function mutations in the Barley gene HvAP2, which has important roles in the regulation of plant height, spike architecture and lodicule development. By studying different hvap2 mutants, the authors revealed the role of HvAP2 in the control floret organ identity and grain development. Also, by reanalyzing a gain-of-function mutant (Zeo1, previously described by this group), the authors confirmed published results on the role of

AP2-like genes in the promotion of floret fate in cereals.
Interestingly the author showed that HvAP2 is required for maternal tissue differentiation and elimination during grain development. The authors provided evidence that HvAP2 may control grain development through the regulation of MADS29 expression, whose inactivation severally affect grain development.
Moreover, the authors studied the interaction between HvAP2 and LAXATUM.A/BLADE-ON-PETIOLE2 (HvBOP2). The results indicated that HvAP2 and HvBOP2 interact to control spike density and lodicule identity.
I think the work is very well organized and presents interesting observations.
I have some comments and suggestions for the authors to consider.

Reviewer 1 Comments for the Author...
Major comments

>About the HvAP2 functions during early floral development
The authors state: “These data support that HvAP2 promotes HvMADS1 expression, potentially explaining the loss of perianth features in gigas1.a and the glume to lemma homeotic transformations in Zeo1.b (lines 206-208)”. The authors show higher MADS1 expression in Zeo1.b by qPCR and in situ, however no change in MADS1 expression is shown in gigas1.a. Therefore, the role of HvMASD1 in the loss of perianth features in gigas1.a is not clear. I understand that the floret organ phenotypes in hvap2 mutants are subtle and it could be hard to see differences in MADS gene expression by qPCR. Did the authors attempt to do MADS1 in situ in Hvap2 mutants?

Author Response: We agree that the relationship between HvAP2 and HvMADS1 may be interesting to follow-up; however, we did not see consistent changes in *HvMADS1* mRNA level in *gigas1.a* by qPCR over three time points (Fig 3D; Fig S8). Based on this, we decided not to pursue in situ hybridisation. Furthermore, we do not think that the in situ experiment would significantly impact our main conclusions or model that HvAP2 can promote *HvMADS1* expression. However, we appreciate the reviewer’s comment about our interpretation and have modified the revised text to remove HvAP2-related changes in *HvMADS1* expression as possibly contributing to certain *gigas1.a* phenotypes (lines 208).

In addition, it is not very clear how RT-qPCR data was normalized and expressed in the graphs. However, there seems to be an increase in MADS1 expression levels from WD3.5 to WD4 in all genotypes (Figure S7). That may indicate that MADS1 expression pattern is controlled independently of HvAP2, which instead may modulate quantitatively MADS1 expression level.

Author Response: We agree that HvAP2 is not the sole regulator of *HvMADS1* expression and that other factors besides HvAP2 must contribute to any age-dependent increases in *HvMADS1* transcript accumulation, especially as *HvMADS1* expression persists in *gigas1.a* at all stages examined. We revised the text in the results and the discussion to emphasise this (line 205-209; lines 405-407). The qPCR was normalised using RQ values calculated by Pfafll method 2^ (-ddCT). One replicate of Bowman at the earliest timepoint was normalised to 1.0 and each other sample normalised to this value (original manuscript line 581). We used the same control genes as in Patil et al (2019) which were ACTIN and PPA2. We now provide more information about the qPCR normalisation in the methods section (lines 596- 600).

>HvAP2 in grain development.
The authors describe the role of HvAP2 in maternal tissue differentiation and elimination during grain development. They suggest JA and MADS29 as putative mediators of HvAP2 functions.
Have the authors tested the effect exogenous JA on grain development in hvap2-2 or gigas1.a mutants?

Author Response: We agree that this is an intriguing experiment. We have considered several ways to tease apart the role of JA in these new roles ascribed to HvAP2. In particular, we are developing genetic resources to address the role of JA in HvAP2 function. However, these resources are some time away from use (> 1 year). Application experiments to assess JA application on grain development involves several design considerations. We are not sure whether the role of JA is important during ovary development or more important role during post-anthesis. We believe that

to do this experiment comprehensively would take better part of a year to conduct different dose curves and temporal applications, followed by data analyses. While we agree that this experiment has great merit, we feel it is better placed in a further study as the relationship between HvAP2 and JA is not central to our major conclusions in this manuscript.

The connection between HvAP2 and MADS29 is very interesting, however the evidence for this interaction is mainly a qPCR result showing a reduced MADS29 expression in hvap2 mutant (gigas1.a). The authors indicated that hvap2 and hvmads29 mutants have similar grain phenotypes, however without results showing a genetic interaction it is difficult to conclude about a connection between these two genes. A cross between Zeo1.b and hvmads29 would be informative.

Author Response: Our proposed interaction between HvAP2 and HvMADS29 is speculative and we have modified the revised text to emphasise this point (lines 330-332). The *hvmads29* line shows severe and earlier phenotype of impaired maternal tissue generation, while *gigas1.a* shows changes in the timing of maternal differentiation/ degradation; so, we speculate that the interaction may be stage and/or tissue dependent and occurs after significant HvMADS29 function in pre-fertilisation tissues. Thus, we predict that the double *hvmads29 gigas1.a* mutant will resemble *hvmads29*. However, we face import and quarantine delays to initiate this cross since the *hvmads29* seed is in Australia while the *Zeo1.b* line is in UK. Factoring in transit and permit time, the timeline for a double mutant for analyses is realistically two years away and we feel that our data should be shared with the scientific community before then. Lack of this cross does not detract from our comparative qPCR, overlapping in situ and mutant data, which together provide a major advance to our understanding of the genes controlling maternal tissue growth, differentiation and elimination in cereals. We are planning work to investigate the molecular nature of the potential interaction as part of a future study.

Have the authors check MADS29 expression in hvap2 mutants by in situ?

Author Response: We decided not to pursue this approach. We feel that the *gigas1.a* phenotype and the modest decrease in *HvMADS29* expression suggests that the *HvMADS29* spatial expression pattern as detected by traditional in situ may not qualitatively differ markedly from wild type. We speculate that the relationship of HvAP2 on HvMADS29 is likely a quantitative and temporal one, best suited to qPCR as presented here. In future experiments we plan to tease apart tissue-specific changes in expression level.

A point not explored by the authors is how HvAP2 controls grain length. It could be interesting to check for changes in cell size and cell number. The shorter grains of Zeo1.b reminds the phenotype of transgenic Arabidopsis expressing an AP2 version that is immune to miR172 and MIM172 constructs described in Ripoll et al., 2015 “microRNA regulation of fruit growth”.

Author Response: We thank the reviewer for this insightful comment. We pursued this suggestion and now include analyses of cell length and width of the pericarp epidermis as well as the adaxial lemma in our revised manuscript. We found that *gigas1.a* adaxial lemma epidermal cell length increased by 52% compared to Bowman (Fig. S1D) suggesting that the 50% increased lemma length may mostly reflect increased cell elongation in *gigas1.a* (lines 116-118). In contrast, *gigas1.a* pericarp epidermal cells were only 16% longer compared to Bowman while *gigas1.a* grain was 47% longer (Fig. S2B), suggesting that changes in *gigas1.a* grain length likely involved both increases in cell size and cell number (lines 128- 130). We did not find differences in cell length in *Zeo1.b* lemmas or pericarp epidermis (Fig. S1D; Fig S2B; lines 136-138), however, wider grain in *Zeo1.b* correlated with 52% increased pericarp cell width (Fig. S2C), suggesting that miR172 regulation of HvAP2 is important to promote medial cell expansion of ovary pericarp in barley (lines 138). Interestingly, though, *gigas1.a* pericarp cells were 23% wider and lemma cells 16% wider compared to Bowman (Fig S1E, S2D, lines 118, 129). We also discuss these data in the context of a conserved role for AP2 in ovary wall cell size (lines 446-449).

>HvAP2 acts downstream of HvBOP2 to control lodicule identity and grain length.
The authors provide genetic interaction data between HvAP2 and HvBOP2 in the control of lodicule identity and spike density. However, I do not see a clear interaction in grain length. Moreover, the authors description of the grain length phenotype appears contradictory:
Line 348 "Zeo1.b lax.a grain length was equivalent to lax.a (p=0.26; Fig. S14)".

Lines 354-355 "Zeo1.b lax.a double mutants also showed striking epistasis in specific features. Double mutants showed Zeo1.b-like spike density (p≤0.001), grain length (p<0.05)" Could the authors clarify this?

Author Response: We apologise for this error. *Zeo1.b lax.a* grain width was equivalent to *lax.a* (p=0.26; Fig. S15). We fixed this in the revised manuscript and explained the phenotype in more detail (line 353).

The interaction HvAP2 and HvBOP2 in lodicule identity is very interesting. The authors also mentioned that "3% of hvap2-2 florets green, filament-like organs with smooth and hairy bract-like regions replaced the lodicules (Fig. 2G, H)". To me, those filament-like organs looks like stamens. Have the authors check for filament-like organs (or stamens) in lodicules of gigas1.a lax.a/+ plants?

Author Response: We did not observe any filament like organs in the *gigas1.a lax.a*/+ plants under our growing conditions. We agree with the reviewer that these organs could represent a stamen-like feature and now include this interpretation in the text (line 174). We thank the reviewer for bringing this possibility to our attention; we feel this supports our proposition that HvAP2 can exclude stamen identity from the lodicule whorl.

Additionally, it is not very clear to me whether HvBOP2 acts upstream or HvBOP2 and HvAP2 act parallelly to control lodicule identity? To clarify, I think it is important to check HvAP2 expression in lax.a mutants.

Author Response: We thank the reviewer for this suggestion. We examined *HvAP2* expression and *HvMir172* expression in Bowman, *lax.a* and *Zeo1.b* whole plants following the floral transition (14 days after germination) and spikes at Waddington stage 3 (awn primdorium, 21 days after germination). These data are presented in Fig S17. We did not detect major changes in *HvAP2* expression in either whole seedlings or spikes of *lax.a*, although we did detect elevated HvAP2 in *Zeo1.b* spikes as previously found. Thus, we suggest that loss of HvBOP2 has no influence on steady-state *HvAP2* transcript levels in these tissues. However, we did detect slightly elevated levels of *HvMir172* mature miRNA in *lax.a* as well as lowered levels in *Zeo1.b*, suggesting that HvAP2 suppresses levels of *HvmiR172* and *lax.a* may permit increased *HvmiR172* expression (lines 359-363). While qPCR can reveal regulatory relationships we are cautious to not over-interpret data from a combination of complex tissues, when we suspect that the functional changes resulting in changes in the lodicule whorl may occur within a small selection of cells at the lodicule/stamen boundary. We recognise that our molecular data for a relationship between HvAP2 and HvBOP2 is incomplete. Accordingly, we have removed from the abstract that our statement that HvBOP2 is upstream of HvAP2 and modified the discussion (lines 431-433); however, our genetic data remains compelling that HvAP2 can suppress effects from a loss of HvBOP2 and can rescue lodicule identity.

Finally, I have a comment about the regulation of grain and hull length. The authors discuss that HvAP2 may modulate grain length by influencing hull length (line 443). However, it seems that Zeo1.b lax.a double mutant has longer lemmas (Fig 6F) but shorter grains (Fig 6J) than the controls. Therefore, based on the authors results elongation of these two organs could actually be uncoupled. Could the author comment on this?

Author Response: We thank the reviewer for raising this intriguing insight. We agree that these data suggest an uncoupling of these two traits and raise the possibility that HvAP2 may shorten grain separately from lemma length regulation. We have included this consideration in the revised text (lines 354) and in the discussion (lines 453-454).

Minor comments

Author Response: We apologise for several inconsistencies in the manuscript and thank the reviewer their careful itemisation of these issues in their review. We fixed all issues and provide extra clarification and modifications as requested in the revised manuscript.

Line 125 "which were decorated with ectopic glume-like hairs". Are these, glume specific or more general bract-like hairs?
Author Response: We believe these are glume-specific based on their similarity to hairs observed on

the glume compared to hairs seen on the rachis or lemma. We now include a second panel to highlight this difference (Fig S1G,H)

Line 130 Remove Fig. S2. Fig1 O is not referred.
Author Response: We fixed these errors and rearranged Fig1 to reflect order in which panels are described in the text (Fig 1O and 1N exchanged).

Line 132 “although gigas1.a lemma width was unchanged compared to Bowman (Fig. S2)”. This is not shown in figure S2. However, Figure S4A shows significant wider lemmas in gigas1.a
Author Response: We apologise for this error. Fig S1A, not Fig S2, shows no change in lemma width in *gigas1.a* compared to Bowman in this experiment; this has been corrected (line 132). The experiment described in Figure S4 did show increased lemma width in *gigas1.a* as pointed out. We adjusted the manuscript text to take this into account (lines 170- 172).

Line 138 I suggest including lax spikes phenotype in the description
Author Response: We now include “lax spikes” in the summary phenotype description on line 139.

Line 144.BOPA1 or BOPA2?
Author Response: These were BOPA2 markers. We modified BOPA1 to BOPA2 label on Supplemental Figure 3 accordingly.

Line 159 "HvCas9". I understand that the authors mean a barley codon optimized Cas9. However, the nomenclature HvCas9 seems not appropriated.
Author Response: We agree that HvCas9 is not an ideal nomenclature. We now call this gene bcoCas9 for **b**arley **c**odon **o**ptimised **Cas9** throughout the manuscript.

Line 162 “deletion (175bp–240bp)”. Should not it be (175bp to 214bp)?
Author Response: We fixed this error (line 163).

Line 176 What do the authors mean by perianth boundary? Lemma-awn boundary?
Author Response: We rephrased this sentence to indicate the boundary between the glume and lemma (outer perianth) specifically (line 178-179).

Line 213 MADS3 and MADS58. The same barley locus number (HORVU3Hr1G026650.1) is indicated for both genes. Besides, if authors refer
to orthologs of Rice MADS3 and MADS58, both are C-class AGAMOUS-like genes (Yamaguchi et al., Plant Cell 2006).
Author Response: We apologise for this error. The correct locus number for *HvMADS58* is HORVU1Hr1G029220.1. We changed the text to fix this error. We agree with the reviewer that both genes are *AGAMOUS*-like and have reworded the sentence to read: “…two AGAMOUS-like genes whose orthologues in Arabidopsis are direct targets of AtAP2” (lines 220-221).

Line 304 I could not see expression in peripheral vascular bundles. Could you please indicate in the figure?
Author Response: We label these structures in the revised figure.

Line 341 “(p<0.04; Fig. 6C–D; Fig. S14)” Should not it be Fig S13?
Author Response: The reviewer is correct. We fixed this error (line 339)

Line 347-348 “S14 and 15”. Should not it be FigS13 and S14 instead?
Author Response: The reviewer is correct. We fixed this error (line 347)

Line 437 “(Fig 1D–F;..)”. Should not it be Fig 1D, O?
Author Response: We have fixed this improper citation to the figure. It now reads (Fig 1C,D,L-N; Fig 2D-E; Fig S4) as appropriate for the text. (line 439)

Line 461 “Fig5G” should be Fig4H.
Author Response: The reviewer is correct. We fixed this error (line 469)

Figure 1N I suggest indicating the position of glumes and lemma-like glumes.

Author Response: We now use white arrows to indicate these organs on Fig1O

Figure 2F Is the scale for GP and hvap2-2 the same? It looks like hvap2-2 is a little bigger.
Author Response: Yes, the scale is the same. The *hvap2-2* spikelets are larger than GP.

Figure 3C I suggest replacing WD7 by WD9. I cannot see very clear differences between lodicules at WD7.
Author Response: We replaced these panels as requested.

Figure 3C and E. Could you indicate how many replicates were performed?
Author Response: We performed three replicates.

Figure 4C-G. Could you indicate how many replicates were performed?
Author Response: We performed five replicates (in addition to 10-14 wax and free hand sections).

Figure 5B. Could you indicate how many replicates were performed?
Author Response: We performed three replicates.

Figure S8 It is labeled as FigS7
Author Response: We fixed this error

Figure S10B What is the purpose of MADS1 sense in situ?
Author Response: We use a sense probe to assess non-specific background staining during the in situ protocol.

Figure S2 and S14. Some discrepancies in Zeo1.b grain width parameters.

Author Response: We have fixed these errors as described above.

Methods
Could you please clarify how RT-qPCR data was normalized? Which gene was used as control gene? How are expression levels express?
Author Response: The qPCR was normalised using RQ values calculated by Pfaffl method 2^ (-ddCT). One replicate of Bowman at the earliest timepoint was normalised to 1.0 and each other sample normalised to this value (original manuscript line 581). We used the same control genes as in Patil et al (2019) which were ACTIN and PPA2. We have provided more information about the qPCR normalisation in the methods section (lines 596-600) and the results (205).

Reviewer 2 Advance Summary and Potential Significance to Field...

This manuscript deals with the functional characterization of HvAP2 in barley. In a previous paper the authors describe the role of HvAP2 in internode regulation. In this manuscript they focused on the role in floret organ determination and grain development and compared it with described functions from orthologous genes from rice and wheat. Several functions of this pleiotropic gene appeared to be conserved, but also new roles in ovule and grain development are reported. A novel part of the work is that part of the network of HvAP2 is elucidated, i.e. an important downstream and a putative upstream regulator have been found and are involved in some of the functions of HvAP2. The results are solid and well described in the figures.

Detailed description of the work:
Searching for mutants of HvAP2, knowing the phenotype of the gain-of function mutant Zeo1.b brought the authors to an ‘old’ mutant (reported in 1962) that overlapped with the HvAP2 locus. This mutant, gigas1.a showed multiple elongated features in the spike and spikelets. Shape of spikelets and grains were more lance-shaped, and awns at the tip of lemmas and glumes affected, suggesting more glume awn-like characteristics of the lemma awn in the mutant. Lodicules develop ectopic bract-like lamina, decorated with ectopic glume-like hairs. In the gain-of HvAP2 mutant Zeo1.b the glumes were often transformed into lemmas, similar to miR172 resistant versions of

TaAP2L in wheat and in line with a lemma identity function for HvAP2. Besides these partial homeotic transformations that mimic a typical A-type mutant, other aspects of female organ and grain development were affected.
Genomic analysis of the gigas1.a mutant revealed that it has a deletion comprising HvAP2 and six other genes. To confirm that the phenotype of the mutant is caused by the deletion of HvAP2, CRISPR/Cas9 mutants were generated and one CRISPR deletion mutant largely phenocopied gigas1.a. Based on these mutants, the authors propose that HvAP2 defines the identity of the outer perianth organs, increases stigmatic papillae branching and widens grain, while also restricting longitudinal growth of spike internodes, spikelets, floret organs and grain.
Searching for MADS box members that could act downstream of HvVAP2 in the florets, revealed HvMADS1, orthologous to the rice LHS1 gene, as a candidate. These two barley genes show an overlap in gene expression and the lhs1 mutant phenotype hints towards a similarity with gigas1.a. HvMADS1 is higher expressed in Zeo1.b, but not affected in gigas1.a (fig 3). This latter is not mentioned in the text but is relevant, because it doesn't fit with the
conclusion that the loss of perianth features and homeotic transformations in gigas1.a can be explained by a reduction of HvMADS1 (line 206-208).
Other MADS-box genes, belonging to the C and D-class could potentially account for the gigas1.a phenotypes in the pistil, lodicule and stigma. This is based on expression of the MADS box genes in the loss and gain-of function mutants.
A detailed phenotypic analysis of WT, Zeo1.b and gigas1.a, including histology (fig 4) was performed showing defects in the nucellus and integument layers of the developing seed. These analysis suggested that HvAP2 promotes degradation of maternal tissues during seed development.
A potentially downstream acting MADS box gene in the grain is HvMADS29, which is orthologous to OsMADS29. Downregulation of OsMADS29 in rice inhibits degradation of maternal tissues, including nucellus, leading to shrunken
seeds. HvMADS29 was downregulated in the gigas1.a mutant, suggesting that it is controlled (directly or indirectly) by HvAP2. The overlap in expression of HvMADS29 and HvAP2 is in developing ovules, in particular in the vascular bundles, but while the expression of HvAP2 goes down during grain development,
HvMADS29 expression is maintained, indicating not a complete epistasis. CRISPR derived hvmads29 mutants showed a number of defects similar to gigas1.a, suggesting that HvAP2 acts upstream of HvMADS29 and is involved in the formation of vascular tissue in the nucellus.
Based on the previous work of the authors (2019) they investigated whether JA is also involved in barley grain development. Two JA-responsive genes JIP23 and JIP60 are downregulated in the gigas1.a mutant.
To search for upstream regulators of HvAP2, the authors found an existing mutant (lax.a) with a deleted HvBOP2 transcription factor gene and displaying multiple defects that phenocopied gigas1.a. The double mutant lax.a Zeo1.b revealed also independent characteristics, indicating a partial epistasis.
This suggests a genetic interaction between HvBOP2 and HvAP2, but I am missing expression studies of HvAP2 in the lax.a mutant background, to understand the transcriptional relationship between the transcription factor and the putative downstream target. Since HvAP2 can be post-transcriptionally regulated by HvmiR172, it might be informative to test the expression of the MiRNA genes as well.

Reviewer 2 Comments for the Author...

I have two suggestions for improvement:

1. I am missing expression studies of HvAP2 in the lax.a mutant background, to understand the transcriptional relationship between the transcription factor and the putative downstream target. Since HvAP2 can be post-transcriptionally regulated by HvmiR172, it might be informative to test the expression of the MiRNA genes as well.

Author Response: We examined *HvAP2* expression and Hvmir172 expression in Bowman, *lax.a* and *Zeo1.b* whole plants following the floral transition (14 days after germination) and spikes at Waddington stage 3 (awn primordium, 21 days after germination). These data are presented in Fig S17. We did not detect major changes in *HvAP2* expression in either whole seedlings or spikes of *lax.a*, although we did detect elevated *HvAP2* in *Zeo1.b* spikes as previously found. Thus, we suggest that loss of HvBOP2 has no influence on steady-state *HvAP2* transcript levels in these

tissues. However, we did detect slightly elevated levels of *Hvmir172* in *lax.a* as well as lowered levels in *Zeo1.b*, suggesting that HvAP2 suppresses levels of *HvmiR172* and *lax.a* may permit increased *HvmiR172* expression (lines 359-363). While qPCR can reveal regulatory relationships we are cautious to not over-interpret data from a combination of complex tissues, when we suspect that the functional changes resulting in changes in the lodicule whorl may occur within a small selection of cells at the lodicule/stamen boundary. We recognise that our molecular data for a relationship between *HvAP2* and *HvBOP2* is incomplete. Accordingly, we have removed from the abstract that our statement that HvBOP2 is upstream of HvAP2 and modified the discussion (lines 431-433); however, our genetic data remains compelling that HvAP2 can suppress effects from a loss of HvBOP2 and can rescue lodicule identity.

2. This manuscript nicely shows some putative downstream genes and a putative upstream transcription factor of the pleiotropic gene HvAP2. Some of the functions are new, but a number has been revealed from orthologous genes in rice, wheat or even Arabidopsis. I would like to see a concluding figure with
the different MADS genes, HvBOP2 and possibly other known factors that illustrates the HvAP2 network and its pleiotropic functions.

Author Response: We have prepared a model as shown in Figure 7.

Reviewer 3 Advance Summary and Potential Significance to Field...

The manuscript by Shoesmith et al. describes the function of HvAP2 controlling floret organ identity, floret boundaries and maternal tissue differentiation and elimination during grain development in Barley. The authors characterize the HvAP2 function by loss-of-function mutants generated by CRISPR-Cas9. Furthermore, expression analyses are performed to observe relationships with MADS domain proteins of the ABCDE model. A detailed analysis is performed on the HvMADS29 protein, a B-sister MADS protein, again using CRISPR loss- of-function alleles were generated and phenotypically analyzed, some overlap in phenotypes is observed compared to the Hvap2 mutants. Finally, generating double mutants, the authors show that HvAP2 functions downstream of HvBOP2.
This story provides nice functional and molecular insight in floret and grain development in Barley. The authors are experts in the theme. All experiments are of high quality and presented in good figures. Writing is excellent.
I do not have any comment or suggestion to improve this manuscript.

Reviewer 3 Comments for the Author...

The authors are experts in the theme. All experiments are of high quality and presented in good figures. Writing is excellent.
I do not have any comment or suggestion to improve this manuscript.

Author Response: We thank the reviewer for this positive feedback.

Second decision letter

MS ID#: DEVELOP/2020/194894

MS TITLE: APETALA2 functions as a temporal factor together with BLADE-ON-PETIOLE2 and MADS29 to control flower and grain development in barley

AUTHORS: Jennifer R Shoesmith, Charles Ugochukwu Solomon, Xiujuan Yang, Laura G Wilkinson, Scott Sheldrick, Ewan van Eijden, Sanne Couwenberg, Laura Pugh, Mhmoud Eskan, Jennifer Stephens, Abdellah Barakate, Sinead Drea, Kelly Houston, Matthew R Tucker, and Sarah M McKim
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

In the revised manuscript, the newly added data analysis and sentences in the main text properly addressed the questions I raised.

Comments for the author

The manuscript is improved after the revision, I just found a few typos:
Line 225 the sentence “AtSHP1 orthologue promotes stigmatic papillae formation in 226 Arabidopsis (Colombo et al., 2010)” should be removed.
Line 286 (PCD(correct to (PCD)
Line 339 FigS14 instead of FigS13?
Line 347 Fig S14, S15 instead of FigS13, S14?
Line 348 FigS14 instead of S13?
Line 351 I don’t agree with the sentence “Grain of gigas1.a lax.a showed the same narrow grain as lax.a (p=0.26; Fig 6I; Fig S14)”. The grains of gigas1.a lax.a seem to be narrower than the parents (FigS15) suggesting an additive effect.
Line 357 FigS15 should be FigS16
Line 361 remove one “however”
Line 363 FigS16 should be FigS17
Line 374 Fig6 should be Fig 7
Line 432 I would expect that HvBOP2 may “repress” the function of other miR172-regulated HvAP2L genes to regulate lodicule identity.