Duplicated antagonistic EPF peptides optimize grass stomatal initiation

Raman Jangra, Sabrina C Brunetti, Xutong Wang, Pooja Kaushik, Patrick J Gulick, Nora A Foroud, Shucai Wang and Jin Suk Lee
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MS TITLE: Duplicated antagonistic EPF peptides optimize grass stomatal initiation

AUTHORS: Raman Jangra, Sabrina C Brunetti, Xutong Wang, Patrick J Gulick, Nora A Foroud, Shucai Wang, and Jin Suk Lee

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.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. 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. Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Jangra et al characterize EPF family peptides in grasses and functionally characterize subset of them. Authors perform refining bioinformatic analysis of EPF family in 6 grass species and based on this analysis identify candidate genes from Brachypodium distachyon and Triticum aestivum for being orthologs of AtEPF1, AtEPF2, and AtEPFL9/STOMAGEN. Authors select genes, which are enriched in leaf developmental stages overlapping with active stomatal development for functional analysis in Arabidopsis. By combining inducible overexpression and cross-species complementation analysis authors show that both Brachypodium and wheat EPF2 orthologs repress initiation of stomatal lineage whereas STOMAGEN orthologs promote stomatal development. Finally, authors treat Brachypodium seedlings with bioactive mature BdEPF2-1/2-2 and BdSTOMAGEN-1 peptides and show that BdEPF2-1/2-2 peptides are sufficient to completely prevent stomatal initiation in Brachypodium whereas BdSTOMAGEN-1 promotes stomatal initiation causing excessive stomata formation. Thus, these peptides are likely to act antagonistically.

Orthologs of EPF1/2 peptides has been studied in rice, barley and wheat (Hughes et al., 2017; Mohammed et al., 2019; Dunn et al., 2019; Caine et al., 2019; Lu et al., 2019). These studies have focused mostly on overexpression analysis and shown that EPF1/2-OE restrict stomatal development and lead to formation of arrested GMCs. Thus, this study describes for the first time that grass (Brachypodium and wheat) EPF2 peptides prevent completely stomatal initiation events similar to EPF2 function in Arabidopsis. It is possible that discrepancies in the current study and previous studies are caused simply by different experimental set up as authors describe nicely in discussion. However, it cannot be ruled out that different grass species may also display species specific molecular strategies in the control of stomatal developmental pathway as has been shown to be the case with orthologs of MUTE (Raissig et., 2017; Wang et al., 2019; Wu et al., 2019).

Studies of grass STOMAGEN has been performed in rice; Yin et al 2017 showed that OsSTOMAGEN/EPFL9-1 loss-of function mutants display reduced stomatal density whereas Lu et al., 2019 showed that its overexpression promotes stomatal development both in rice and Arabidopsis. Results of this manuscript are in line with previous studies regarding grass STOMAGEN orthologs.

Although effects of EPF peptides in grass stomatal development has been extensively studied, this study provides additional information beyond previous studies and refines the current view on the regulation of grass stomatal development.

Comments for the author

This is carefully planned work with rigorous experimental set up. Manuscript is well written and conclusions are solid.

Minor comments:

- Page 11: Authors wrote" However, unlike Arabidopsis, we also found that application of either MBdEPF2-1 or MBdEPF2-2 peptide failed to induce any obvious change to other nonstomatal epidermal cells, such as silica cells in veins and hair cells, although the generation of stomata and stomatal precursors were completely blocked." Please explain how does this differ from effects in Arabidopsis non-stomatal cells.

- It is interesting that treatment with BdSTOMAGEN-1 peptide leads to unusual subsidiary cell morphologies. Please show whether this phenotype is suppressed by BdEPF2 treatment.

- Page 12: " …, our results also suggest that BdSTOMAGEN regulates several stages of stomatal development and patterning in grasses." This is an exciting possibility. However, since all the data in Brachypodium is currently based on peptide treatments, it is possible that unusual subsidiary cell morphologies are caused by faulty peptide dosage, incorrect timing or domain of peptide exposure Please discuss these possibilities or alternatively add supporting data such as stage specific reduction of BdSTOMAGEN-1 levels (for example by using amiRNA).

- Please explain more clearly what is currently known from grass stomata controlling EPFs: for example, Yin et al 2017 (which studied rice EPFL9) is referred only once and in incorrect context: Page 13, second paragraph: “Recent studies for at least one of these two AtEPF1/AtEPF2-like genes in some grass species suggest that they have a role in controlling stomatal differentiation”. Please modify to be in line with current literature.

Potential extensions of the study:

This work would greatly benefit from loss of function analysis of BdEPF2-1/2-2 and especially BdSTOMAGEN-1. These experiments would clarify the roles of these genes but also peptide-receptor signaling in the stomatal lineage in grasses. Putative novel functions of BdSTOMAGEN-1 during stomatal development could be precisely defined by stage specific rescue of BdSTOMAGEN-1 LOF mutant for example by using already available BdSPCH and BdMUTE promoters (Raissig et al., 2016, Raissig et al., 2017).

Reviewer 2

Advance summary and potential significance to field

The EPF/EPFL family extracellular peptides enable cell-to-cell communications and regulate multiple aspects of plant development. In their manuscript, Jangra et al. describe function of several EPF peptides during stomata formation in two grasses: the model grass Brachypodium distachyon and in wheat. Authors created a phylogenetic tree of EPF/EPFLs from Arabidopsis, Brachypodium, and wheat, overexpressed grass EPFs in Arabidopsis, performed complementation of Arabidopsis mutants by grass EPFs and analyzed stomata formation in Arabidopsis and Brachypodium after treatment with exogenous grass EPFs. Overall, this work demonstrated that EPFs function very similar in dicots and monocots. The main exception is that, while in Arabidopsis EPF1 and EPF2 function during distinct stages of stomata formation, in Brachypodium and wheat all EPFs regulate stomata initiation and none are involved in the control of their differentiation. This manuscript, while not groundbreaking, is a valuable step forward in understanding the role of EPFs in the stomata formation in monocots.

Comments for the author

The experiments are well-designed and properly executed. The manuscript contains a lot of high-quality images and figures are easy to understand. Conclusions are supported by findings. The paper is logically written but has a lot of grammar and spelling mistakes.

Recommendations:

1. Please proofread manuscript more carefully.

2. In fig 1. a branch corresponds to AtEPFL6 is absent on the phylogenetic tree. It is difficult to understand where AtEPFL6 is located.

3. I found dots indicating stomata on Fig 4 and 5 to be misleading.
Because these dots are in a middle of a pavement cell, the first impression one gets is that these dots highlight specific pavement cells. Maybe dots can be replaced with arrows?

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First revision

Authors' Responses to Reviewers' Comments (**in bold**)

Reviewer #1 Advance Summary and Potential Significance to Field:

Jangra et al characterize EPF family peptides in grasses and functionally characterize subset of them. Authors perform refining bioinformatic analysis of EPF family in 6 grass species and based on this analysis identify candidate genes from Brachypodium distachyon and Triticum aestivum for being orthologs of AtEPF1, AtEPF2, and AtEPFL9/STOMAGEN. Authors select genes, which are enriched in leaf developmental stages overlapping with active stomatal development for functional analysis in Arabidopsis. By combining inducible overexpression and cross-species complementation analysis authors show that both Brachypodium and wheat EPF2 orthologs repress initiation of stomatal lineage whereas STOMAGEN orthologs promote stomatal development. Finally, authors treat Brachypodium seedlings with bioactive mature BdEPF2-1/2-2 and BdSTOMAGEN-1 peptides and show that BdEPF2-1/2-2 peptides are sufficient to completely prevent stomatal initiation in Brachypodium whereas BdSTOMAGEN-1 promotes stomatal initiation causing excessive stomata formation. Thus, these peptides are likely to act antagonistically.

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Although effects of EPF peptides in grass stomatal development has been extensively studied, this study provides additional information beyond previous studies and refines the current view on the regulation of grass stomatal development.

We thank Reviewer 1 for acknowledging the importance and impact of our findings to the field.

Comments for the Author:

This is carefully planned work with rigorous experimental set up. Manuscript is well written and conclusions are solid.

We thank Reviewer 1 for the very positive comments.

Minor comments:

- Page 11: Authors wrote" However, unlike Arabidopsis, we also found that application of either MBdEPF2-1 or MBdEPF2-2 peptide failed to induce any obvious change to other nonstomatal epidermal cells, such as silica cells in veins and hair cells, although the generation of stomata and stomatal precursors were completely blocked." Please explain how does this differ from effects in Arabidopsis non-stomatal cells.

During epidermal development in both Arabidopsis and Brachypodium, a protodermal cell first makes a fate decision of whether or not to be the meristemoid mother cell (small cells

in stomatal files of the Brachypodium leaf), which undergoes asymmetric cell division that produces two daughter cells: one cell that acts as a stomatal precursor and another that differentiates as a pavement cell. AtEPF2 inhibits this asymmetric cell division that initiates the stomatal cell lineage and, consistent with its role in Arabidopsis, either *AtEPF2* overexpression or application of bioactive AtEPF2 peptide exhibits an epidermis solely composed of pavement cells (Hara et al. 2009 Plant Cell Physiol; Hunt and Gray 2009 Curr Biol; Lee et al. 2012 Genes Dev).

We found that application of either mature BdEPF2-1 or BdEPF2-2 peptides to Brachypodium seedlings completely blocks the formation of stomata and stomatal precursors, which resembles the phenotype of *AtEPF2* overexpression (or application of bioactive AtEPF2 peptide) in Arabidopsis. However, blocking of these asymmetric entry divisions in stomatal cell files of the Brachypodium did not result in a leaf epidermis composed entirely of pavement cells, even in the stomatal cell files. This suggests that the fate of non-stomatal epidermal cells (e.g., hair cells) is not affected by BdEPF2 peptides and stomatal cell fate establishment and asymmetric entry division most likely happens after asymmetric cell division that generates other non-stomatal cell types in the Brachypodium. We reworded our sentence on page 11 for a better explanation.

- It is interesting that treatment with BdSTOMAGEN-1 peptide leads to unusual subsidiary cell morphologies. Please show whether this phenotype is suppressed by BdEPF2 treatment.

This is an excellent suggestion. To determine potential, additional relationships between BdEPF2 and BdSTOMAGEN during stomatal development in Brachypodium, we have performed a series of combined bioactive BdEPF2-2 and BdSTOMAGEN-1 peptide application experiments using Brachypodium wild-type seedlings. Exogenous application of mature BdSTOMAGEN-1 (MBdSTOMAGEN-1) peptide promoted stomatal linage cell fate, causing increased stomatal density and clustering. As Reviewer 1 points out, MBdSTOMAGEN-1 treatment also led to patterning and/or fate defects in other epidermal cell types, such as subsidiary cells. As shown in Fig. S11, we found that simultaneous application of MBdSTOMAGEN-1 peptide with increasing concentration of MBdEPF2-2 peptide decreased stomatal differentiation in a dose-dependent manner, but increasing the concentration of MBdEPF2-2 peptide did not suppress the phenotype of unusual subsidiary cell morphologies caused by MBdSTOMAGEN-1 application. These results support our finding that BdEPF2 and BdSTOMAGEN-1 peptides with opposing activities function together at the early stage of stomatal development (stomatal initiation) to optimize stomatal density, but likely not in other aspects of stomatal development (e.g., subsidiary cell formation) in Brachypodium. These new data are presented as Fig. S11 and described in the revised text.

- Page 12: " …, our results also suggest that BdSTOMAGEN regulates several stages of stomatal development and patterning in grasses." This is an exciting possibility. However, since all the data in Brachypodium is currently based on peptide treatments, it is possible that unusual subsidiary cell morphologies are caused by faulty peptide dosage, incorrect timing or domain of peptide exposure Please discuss these possibilities or alternatively add supporting data such as stage specific reduction of BdSTOMAGEN-1 levels (for example by using amiRNA).

As we presented in Fig. 5, Fig. 6 and Fig. S10, application of either bioactive mature BdEPF2-1/2-2 or Bd2g53661 peptides (produced and treated exactly in the same way as MBdSTOMAGEN-1) to Brachypodium seedlings did not result in an epidermis with unusual subsidiary cell morphologies. Together with our previous reports on well-studied Arabidopsis EPF peptides, which showed that the application of each bioactive EPF peptide triggers a unique response consistent with its specific role at a distinct stage of stomatal development (e.g., Lee et al. 2012 Genes Dev; Lee et al. 2015 Nature), it is unlikely that abnormal subsidiary cells found in the MBdSTOMAGEN-1 treated Brachypodium leaf are caused by factors other than its own activity.

Unlike Arabidopsis, grass stomatal development is highly organized, temporally and spatially, so each major developmental stage can be easily monitored by imaging the cells on the leaf epidermis from bottom to tip. Therefore, we have examined in detail how subsidiary cell defects on BdSTOMAGEN-1 treated Brachypodium leaves arise by observing cells at subsidiary

cell formation stage of stomatal development in Brachypodium. Please note that we did not find any defects in later stages of stomatal development, such as guard mother cell division and stomatal differentiation by MBdSTOMAGEN-1 application. As shown in Fig. S9B, treatment of MBdSTOMAGEN-1 to Brachypodium seedlings displayed unusual subsidiary cell formation either by spanning of multiple smaller daughter cells (the results of ectopic asymmetric entry divisions) which will become stomatal precursors, or by producing extra irregular asymmetric divisions in neighboring cells of stomatal precursors. This result indicates that BdSTOMAGEN-1 may have an additional role in promoting asymmetric divisions to produce both stomatal precursors and subsidiary cells, and that unusual subsidiary cells found on the epidermis of MBdSTOMAGEN-1 treated Brachypodium seedlings likely arise from faulty asymmetric divisions.

We agree with Reviewer 1 that further in-depth experiments, such as testing the effects of stage-specific reduction of BdSTOMAGEN (as suggested by Reviewer 1), would be very helpful in convincingly demonstrating our claim for the potential roles of BdSTOMAGEN in regulating additional aspects of grass stomatal development. Such experiments are technically challenging, and we believe they are out-of-scope for this manuscript since our work focuses on grass EPF peptides controlling stomatal initiation. In addition to providing new data (Fig. 9B), we have included some of this discussion in our revised manuscript as suggested.

- Please explain more clearly what is currently known from grass stomata controlling EPFs: for example, Yin et al 2017 (which studied rice EPFL9) is referred only once and in incorrect context: Page 13, second paragraph: “Recent studies for at least one of these two AtEPF1/AtEPF2-like genes in some grass species suggest that they have a role in controlling stomatal differentiation”. Please modify to be in line with current literature.

We agree that more specific information on EPFs known to control grass stomatal development would help readers. Thus, as suggested by Reviewer 1, we have added information to the revised text for clarity. The work of Yin et al is now referred to in three places, pages 4, 14 and 17, in the context of discussion for the previous work on STOMAGEN/EPFL9 orthologs. Thank you very much for the constructive comment and suggestion.

Potential extensions of the study:
This work would greatly benefit from loss of function analysis of BdEPF2-1/2-2 and especially BdSTOMAGEN-1. These experiments would clarify the roles of these genes but also peptide-receptor signaling in the stomatal lineage in grasses. Putative novel functions of BdSTOMAGEN-1 during stomatal development could be precisely defined by stage specific rescue of BdSTOMAGEN-1 LOF mutant for example by using already available BdSPCH and Bd*M*UTE promoters (Raissig et al., 2016, Raissig et al., 2017).

We agree with Reviewer 1 that it would be the most exciting future direction to gain insight into the signaling specificity of the peptide signaling during grass stomatal development. For example, dissecting BdSTOMAGEN functions in specific stomatal lineage cell types, presumably by cell-type-specific rescue experiments using stomatal lineage cell-type-specific promoters in Brachypodium (as suggested by Reviewer 1), would provide clarity into the specificity of EPF signaling during grass (Brachypodium) stomatal development.

A good way to approach this investigation would be to produce transgenic plants with the expression of the *EPF* genes under the regulation of grass stomatal developmental stage specific genes. However, dissecting individual EPF signaling events in specific stomatal lineage cell types in Brachypodium remains a future challenge, which is out of the scope of this manuscript. We have included some of this discussion in our revised manuscript.

Reviewer #2 Advance Summary and Potential Significance to Field:

The EPF/EPFL family extracellular peptides enable cell-to-cell communications and regulate multiple aspects of plant development. In their manuscript, Jangra et al. describe function of several EPF peptides during stomata formation in two grasses: the model grass Brachypodium distachyon and in wheat. Authors created a phylogenetic tree of EPF/EPFLs from Arabidopsis,

Brachypodium, and wheat, overexpressed grass EPFs in Arabidopsis, performed complementation of Arabidopsis mutants by grass EPFs, and analyzed stomata formation in Arabidopsis and Brachypodium after treatment with exogenous grass EPFs. Overall, this work demonstrated that EPFs function very similar in dicots and monocots. The main exception is that, while in Arabidopsis EPF1 and EPF2 function during distinct stages of stomata formation, in Brachypodium and wheat all EPFs regulate stomata initiation and none are involved in the control of their differentiation. This manuscript, while not groundbreaking, is a valuable step forward in understanding the role of EPFs in the stomata formation in monocots.

We thank Reviewer 2 for recognizing our findings as valuable.

Comments for the Author:

The experiments are well-designed and properly executed. The manuscript contains a lot of high-quality images and figures are easy to understand. Conclusions are supported by findings. The paper is logically written but has a lot of grammar and spelling mistakes.

Thank you very much for your kind words and we apologize for the oversight.

Recommendations:
1. Please proofread manuscript more carefully.

We have asked two native English speakers to carefully go over our manuscript to improve the presentation. Thank you for your suggestion.

2. In fig 1. a branch corresponds to AtEPFL6 is absent on the phylogenetic tree.
It is difficult to understand where AtEPFL6 is located.

The branch for AtELPL6 was present but difficult to see because it was printed as AtCHALLAH/AtEPFL6. Thus, we have changed the labeling to AtCHALLAH (AtEPFL6) to make it more visible. We made a similar change to AtSTOMAGEN (AtEPFL9). Thank you for pointing this out.

3. I found dots indicating stomata on Fig 4 and 5 to be misleading. Because these dots are in a middle of a pavement cell, the first impression one gets is that these dots highlight specific pavement cells. Maybe dots can be replaced with arrows?

This has been changed as Reviewer 2 suggested.

4. Wasn't TaEPF2-1 or TaEPF2-2 called previously TaEPF1 by Dunn et al. in "Reduced stomatal density in bread wheat leads to increased water-use efficiency"? Please make it clear in the text. I can see the logic why both genes are called EPF2 by authors, but that will make it confusing to refer to them in the future.

While our manuscript was in preparation, Julie Gray's group reported that overexpression of *AtEPF1*- and *AtEPF2*-like genes in wheat, named as *TaEPF1* and *TaEPF2*, reduced stomatal density, and they both behaved like overexpression of *AtEPF1* resulting in a leaf epidermis with an access number of non-stomatal cells (Dunn et al., 2019 Journal of Experimental Botany). In contrast, we found that *TaEPF1* and *TaEPF2* are more similar in sequence to *AtEPF2* than *AtEPF1* (Table S2). Consistent with the sequence comparison and results from a series of our functional analyses of two *AtEPF1/AtEPF2*-like genes in the wheat relative *Brachypodium disstachyon*, we demonstrated their roles in the early stage of stomatal development where AtEPF2 functions. Overexpression of both wheat *AtEPF1* and *AtEPF2* homologous led to a severe decrease in stomatal and non- stomatal cell density. In addition, detailed phenotypic analysis of overexpression or treatment of two bioactive AtEPF1/AtEPF2-like peptides (named as BdEPF2-1 and BdEPF2-2 in the manuscript) during different stages of stomatal development in Brachypodium provided further mechanistic insight into how duplicated BdEPF2 peptides control the stomatal initiation. We thus in the previous version of the manuscript decided to name wheat AtEPF1/AtEPF2 homologues as TaEPF2- 1 and TaEPF2-

2 (similar to BdEPF2-1 and BdEPF2-2 named for AtEPF1/AtEPF2 homologous in Brachypodium).

We, concur with Reviewer 2 that renaming the previously reported wheat AtEPF1/AtEPF2 homologous, would confuse readers. Therefore, as suggested by Reviewer 2, we have revised the manuscript using the previously published *T. aestivum* gene names and reworded our text to explain this point more clearly. We did maintain the gene names BdEPF2-1 and BdEPF2-2 for the reasons explained above. Thank you very much for the constructive comment and suggestion.

Second decision letter

MS ID#: DEVELOP/2021/199780

MS TITLE: Duplicated antagonistic EPF peptides optimize grass stomatal initiation

AUTHORS: Raman Jangra, Sabrina C Brunetti, Xutong Wang, Pooja Kaushik, Patrick J Gulick, Nora A Foroud, Shucai Wang, and Jin Suk Lee
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 2

Advance summary and potential significance to field

The EPF/EPFL family extracellular peptides enable cell-to-cell communications and regulate multiple aspects of plant development. In their manuscript, Jangra et al. describe function of several EPF peptides during stomata formation in two grasses: the model grass Brachypodium distachyon and in wheat. Authors created a phylogenetic tree of EPF/EPFLs from Arabidopsis, Brachypodium, and wheat, overexpressed grass EPFs in Arabidopsis, performed complementation of Arabidopsis mutants by grass EPFs and analyzed stomata formation in Arabidopsis and Brachypodium after treatment with exogenous grass EPFs. Overall, this work demonstrated that EPFs function very similar in dicots and monocots. The main exception is that, while in Arabidopsis EPF1 and EPF2 function during distinct stages of stomata formation, in Brachypodium and wheat all EPFs regulate stomata initiation and none are involved in the control of their differentiation. This manuscript, while not groundbreaking, is a valuable step forward in understanding the role of EPFs in the stomata formation in monocots. The experiments are well-designed and properly executed. The manuscript contains a lot of high-quality images and figures are easy to understand. The paper is logically written.

Comments for the author

In the revision, authors successfully addressed all concerns raised by the reviewers.