Heterologous expression of a lycophyte protein enhances angiosperm seedling vigor

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MS TITLE: Heterologous expression of a lycophyte protein enhances angiosperm seedling vigor

AUTHORS: Samuel W.H. Koh, Carlisle Bascom, Eduardo Berenguer, Gwyneth Ingram, Mark Estelle, and Christian Hardtke

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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

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

The manuscript by Koh et al. further develops the study of sub-functionalization of the BRX family proteins that was reported earlier by the authors. In this work heterologous expression of various BRX genes from different phylogenetic groups (including non-seed plants) was used to evaluated their effect in Arabidopsis. By using a lycophyte shorter version of BRX missing the regulatory phosphosite and with shorter linkers between the BRX domains, the authors observed a dominant, growth-promoting effect in Arabidopsis. It is argued that such BRX variants could serve as biotechnological tools to promote plant growth.
In general, the manuscript is sound and illustrates nicely how such a heterologous expression strategy can learn more about the true nature or original function of protein families. However, I am not sure whether Development is the right platform for this story, because it does not “provide novel perspectives that advance our understanding of development”.

Comments for the author

- Heterologous expression of BRX-like genes in Arabidopsis is performed here exclusively using the AtBRX promoter. The phenotypic read-out used to decide if complementation was achieved is in many cases the disappearance of the defect in the protophloem “and/or” (in other cases?) the root elongation growth. It is however, not clear how both phenotypes are correlated. In other words, is it possible to only complement for one of the two? In the introduction it is stated that the protophloem differentiation defect is causative for the reduced root growth vigor. In this context, I do not understand how for instance the AmbBRXL1-CITRINE fusion protein codon-optimized for Arabidopsis can (partially) complement the root growth “or” protophloem defects. In view of a causative relationship you would expect both traits would be complemented.
- The AtBRX promoter is presented here as protophloem specific, however, as can be deduced from the pictures provided in Figure S2, promoter activity can be seen in the root stem cell niche as well (See panels Q, R and S). In view of the importance of this region for root growth in which over-accumulation of auxin can result in short-root phenotypes combined with the idea formulated by the authors that BRX proteins might act as versatile scaffolds to recruit other proteins to the plasma membrane thereby influencing phytohormone transport, it is not clear why the stem cell niche expression/activity of BRX is left undiscussed or even disregarded.
- Lines 184 – 185: Mature SmBRX transgenic embryos are bigger than wildtype embryos. The expression of AtBRX during embryogenesis by Scacchi et al. (2009) (not so clearly by Bauby et al., 2007) indicates early and general expression in all embryonic cells. This indicates a general cellular effect which could be a higher cell proliferation activity from early embryogenesis onwards resulting in a larger embryo size and enlarged seed size. This phenotype is, as indicated by the authors in the discussion, reminiscent to the enlarged embryos characteristic of abi5 mutant embryos and suggests an effect on ABA sensitivity.
Unfortunately, the confocal images of the calcofluor white stained embryo of the SmBRX transgenic seed is less clear at the level of the cellular pattern compared to the Col-O embryo (Figure S4). A better picture allowing a view of the cellular pattern in the SmBRX embryo would be helpful. Furthermore, mutants in ABA2 and in other genes involved in ABA biosynthesis (ABA1 and NCED6) all show very similar phenotypes and produce larger seeds indicating ABA synthesis and accumulation is relevant to embryo and seed size. The enlarged seed size of aba2-1 was shown to result from enhanced cell division rather than cell elongation in the embryo. In conclusion, to provide a better insight into the mode of action of the SmBRX gain-of-function, a more detailed study at the cellular level of the enlarged embryos (cell size or cell number affected?) and ABA measurements and/or ABA treatments to complement the phenotype should be addressed.
- Lines 88 – 89: A final conclusion is that SmBRX expression in A. thaliana wildtype substantially enhances “seed vigor”. I am not sure whether this is a correct statement in view of the definition of “seed vigor”: a complex physiological trait that is necessary to ensure the rapid and uniform emergence of plants in the field, essentially including the seed longevity, the tolerance of environmental stresses by germination, and the ability to withstand prolonged storage. I did not found elements in the present manuscript that would specifically indicate the effect on these kind of traits.
- Lines 162 – 163: Adult root system growth is said to be monitored using “rhizotrons”. The term rhizotron is misleading here as this refer to tools for making nondestructive, repeated observations and measurements of root systems that are generally foreseen with transparent viewing surfaces. As far I understood from reading the Material & Methods a destructive method (with removal of the root systems) composed of tubes were used which is clearly not a rhizotron set-up.

- Lines 23 – 24: “Angiosperm seeds develop inside fruits and are connected to the mother plant through vascular tissues”. This sentence in the abstract seems to be prepare the reader for a story on seed development and/or vascular development which is not the case.

Reviewer 2

Advance summary and potential significance to field

Koh et al., demonstrate that the heterologous expression of BRX from the lycophyte Selaginella moellendorffii in A. thaliana wildtype background leads to enhanced root growth vigor and substantial increase in seed and embryo size.
They demonstrate this finding is consistent and reproducible over a range of conditions. Finally they engineered an AtBRX variant that resembled the Selaginella moellendorffii protein and this mirrors the root growth vigor, and size of the seed and embryo phenotypes. This final experiment allowed them to conclude that it was removal of the regulatory phosphosite in conjunction with a size reduction in the linker between the BRX domains that resulted in the growth-promoting effects.
Although it was known previously that linker length influences root growth vigor based on studies in Arabidopsis this study takes a novel approach by studying gene variants from diverse species to understand the role of the linker sequence in more detail. Combined, I think this approach and interesting findings make this study a good advance in our understanding of these key genes.
The manuscript was very well structured and written. The results and figures were excellent and the text and supplementary figures were of very high quality.
I thank the authors for preparing such a high quality submission.

Comments for the author

I have five general comments and a small number of specific comments.
1. As mentioned briefly in the discussion the study previously by the authors Beuchat et al., 2010a already demonstrated that a deletion in the linker between the BRX-domains could confer increase root vigor. It might be useful to introduce this in slightly more detail in the introduction.
2. Given how much of the study requires comparison between gene structure especially of the linker sequences between species I think it might be useful to include a text figure with schematics of the genes and potentially alignments of the linker sequences. This information is included in supplementary information but may help orientate readers if it is included in the text too.
3. From the evolutionary analysis I think including fern sequences in the alignment would be useful. It was slightly unusual to omit them given the extent of sampling in other lineages.
4. Given the authors conclusions about the importance of linker length it might have been good to investigate if there are any trends in linker length in different groups of land plants based on the main sequence alignment they carried out.
5. MpBRXL1 has a short linker sequence but doesn’t increase root vigor.
Does this help you narrow down which portion of the linker sequence is important for root vigor rather than simply the length of it – this might be something to mention in the discussion?
Specific comments Line 26: “non-vascular bryophytes and non-seed lycophytes” what about ferns
Line 41: “culminated in the angiosperms” that is a very angiosperm centric view and also makes it sound like evolution has now just reached its end point.
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Line 50: “green lineage” it might be good to state this group explicitly
Line 104: “basal angiosperm” early diverging lineage of angiosperms

First revision

Author response to reviewers' comments

"Reviewer 1 Advance summary and potential significance to field The manuscript by Koh et al. further develops the study of sub-functionalization of the BRX family proteins that was reported earlier by the authors. In this work heterologous expression of various BRX genes from different phylogenetic groups (including non-seed plants) was used to evaluated their effect in Arabidopsis. By using a lycophyte shorter version of BRX missing the regulatory phosphosite and with shorter linkers between the BRX domains, the authors observed a dominant, growth-promoting effect in Arabidopsis. It is argued that such BRX variants could serve as biotechnological tools to promote plant growth.In general, the manuscript is sound and illustrates nicely how such a heterologous expression strategy can learn more about the true nature or original function of protein families. However, I am not sure whether Development is the right platform for this story, because it does not “provide novel perspectives that advance our understanding of development”."

We would like to thank the reviewer for the overall positive assessment and the constructive comments.

"Reviewer 1 Comments for the author
- Heterologous expression of BRX-like genes in Arabidopsis is performed here exclusively using the AtBRX promoter. The phenotypic read-out used to decide if complementation was achieved is in many cases the disappearance of the defect in the protophloem “and/or” (in other cases?) the root elongation growth. It is, however, not clear how both phenotypes are correlated. In other words, is it possible to only complement for one of the two? In the introduction it is stated that the protophloem differentiation defect is causative for the reduced root growth vigor. In this context, I do not understand how for instance the AmbBRXL1-CITRINE fusion protein codon-optimized for Arabidopsis can (partially) complement the root growth “or” protophloem defects. In view of a causative relationship you would expect both traits would be complemented."

Yes, this is a very keen observation, which highlights the highly quantitative aspect of the protophloem mutant phenotype. Briefly, please note that the appearance of “gaps” reflects a lack of cell wall build up, most likely reduced pectin, that serves as a proxy for dysfunctional protophloem. Importantly, the protophloem mutants display quite some between-individual variation, in the sense that sometimes one can observe defects in both, sometimes only in one, and sometimes in none of the protophloem cell files (see, for example, Figure 5A). The root length is typically associated with this severity, i.e. individuals that display a visible defect in only one strand typically have a milder phenotype than those that display defects in both strands at a given point of observation (see also Breda et al. 2017, PNAS). For clarity we now extended our pertinent comment in the introduction (line 91).

"- The AtBRX promoter is presented here as protophloem specific, however, as can be deduced from the pictures provided in Figure S2, promoter activity can be seen in the root stem cell niche as well (See panels Q, R and S). In view of the importance of this region for root growth in which over-accumulation of auxin can result in short-root phenotypes combined with the idea formulated by the authors that BRX proteins might act as versatile scaffolds to recruit other proteins to the plasma membrane thereby influencing phytohormone transport, it is not clear why the stem cell niche expression/activity of BRX is left undiscussed or even disregarded."

Again, a very keen observation, indeed AtBRX promoter activity can sometimes be detected in the root stem cell niche. However, we know that expression of AtBRX under control of the highly protophloem-specific CVP2 or CLE45 promoters fully rescues the brx mutant, and so far we have not found any functional significance of AtBRX expression in the stem cell niche. We do however discuss the stem cell niche expression now (line 267), and also added the important point that the AtBRX gene body contributes substantially to the AtBRX expression pattern (Koh et al. 2021, Plant Cell). Because of these various confounding factors, we decided to monitor SmBRX expression in detail. These experiments show that transgenic SmBRX is expressed in the (phloem) vasculature throughout the embryo, including the cotyledons, and also in the shoot apical meristem in early stages. These new findings are shown in Figure 4C-H and described and discussed in the text (lines 196 & 270).

"- Lines 184 – 185: Mature SmBRX transgenic embryos are bigger than wildtype embryos. The expression of AtBRX during embryogenesis by Scacchi et al. (2009) (not so clearly by Bauby et al., 2007) indicates early and general expression in all embryonic cells. This indicates a general cellular effect which could be a higher cell proliferation activity from early embryogenesis onwards resulting in a larger embryo size and enlarged seed size. This phenotype is, as indicated by the authors in the discussion, reminiscent to the enlarged embryos characteristic of abi5 mutant embryos and suggests an effect on ABA sensitivity. Unfortunately, the confocal images of the calcofluor white stained embryo of the SmBRX transgenic seed is less clear at the level of the cellular pattern compared to the Col-O embryo (Figure S4). A better picture allowing a view of the cellular pattern in the SmBRX embryo would be helpful. Furthermore, mutants in ABA2 and in other genes involved in ABA biosynthesis (ABA1 and NCED6) all show very similar phenotypes and produce larger seeds indicating ABA synthesis and accumulation is relevant to embryo and seed size. The enlarged seed size of aba2-1 was shown to result from enhanced cell division rather than cell elongation in the embryo. In conclusion, to provide a better insight into the mode of action of the SmBRX gain-of-function, a more detailed study at the cellular level of the enlarged embryos (cell size or cell number affected?) and ABA measurements and/or ABA treatments to complement the phenotype should be addressed."

See above, the expression pattern is more specific than implied by the initial GUS reporter constructs. The latter might be somewhat misleading because i) one misses the contribution of the AtBRX gene body (Scacchi et al.), and ii) is a promoter trap within a large intron of the 5'UTR (also see response to point above). We have now determined the SmBRX expression pattern in embryos and quantified the cellular effects. Consistent with specific expression in the vasculature, we observe an increase specifically in stele width and vascular file number. These new data are displayed in Figure 4I-J and described and discussed in the text (lines 201 & 270).
Yes, the initially chosen image of the SmBRX embryo was unfortunately blurry, here we had flattened the embryos (in order to open the cotyledons) to best display the size difference. We have now replaced the two images with clearer examples from "non-squeezed" embryos (Figure 4A-B). Nevertheless, we did not succeed in a full scale cellular analysis, for example by segmentation. According to expert colleagues in the field who we consulted, this is not trivial for embryos and unrealistic at larger scale. We hope that our conventional cell counts across optical cross sections are satisfactory (Figure 4J).
Regarding ABA, we do not have the technical capabilities to measure ABA levels, but we monitored ABA response. Again, SmBRX transgenics kept their relative advantage (Figure S4A), indicating that ABA response is a priori normal. This new finding is described and discussed in the text (lines 163 & 281).

"- Lines 88 – 89: A final conclusion is that SmBRX expression in A. thaliana wildtype substantially enhances "seed vigor". I am not sure whether this is a correct statement in view of the definition of "seed vigor": a complex physiological trait that is necessary to ensure the rapid and uniform emergence of plants in the field, essentially including the seed longevity, the tolerance of environmental stresses by germination, and the ability to withstand prolonged storage. I did not found elements in the present manuscript that would specifically indicate the effect on these kind of traits."

Indeed, thank you for spotting this, we became aware of the more narrow definition when drafting the manuscript and replaced "seed vigor" by "seedling vigor", but overlooked this particular instance, which has now been corrected (line 97).

"- Lines 162 – 163: Adult root system growth is said to be monitored using "rhizotrons". The term rhizotron is misleading here as this refer to tools for making nondestructive, repeated observations and measurements of root systems that are generally foreseen with transparent viewing surfaces. As far I understood from reading the Material & Methods a destructive method (with removal of the root systems) composed of tubes were used which is clearly not a rhizotron set-up."

Yes, for lack of a better expression we used rhizotron, we now replaced it with "tubes" (line 173).

"- Lines 23 – 24: "Angiosperm seeds develop inside fruits and are connected to the mother plant through vascular tissues". This sentence in the abstract seems to be prepare the reader for a story on seed development and/or vascular development which is not the case."

Indeed, we now highlight that SmBRX expression led to enhanced vascular tissue proliferation to make that link (line 32).

"Reviewer 2 Advance summary and potential significance to field Koh et al., demonstrate that the heterologous expression of BRX from the lycophyte Selaginella moellendorffii in A. thaliana wildtype background leads to enhanced root growth vigor and substantial increase in seed and embryo size. They demonstrate this finding is consistent and reproducible over a range of conditions. Finally they engineered an AtBRX variant that resembled the Selaginella moellendorffii protein and this mirrors the root growth vigor, and size of the seed and embryo phenotypes. This final experiment allowed them to conclude that it was removal of the regulatory phosphosite in conjunction with a size reduction in the linker between the BRX domains that resulted in the growth-promoting effects. Although it was known previously that linker length influences root growth vigor based on studies in Arabidopsis this study takes a novel approach by studying gene variants from diverse species to understand the role of the linker sequence in more detail. Combined, I think this approach and interesting findings make this study a good advance in our understanding of these key genes. The manuscript was very well structured and written. The results and figures were excellent and the text and supplementary figures were of very high quality. I thank the authors for preparing such a high quality submission."

Thank you for the overall positive assessment and the constructive comments!

"Reviewer 2 Comments for the author I have five general comments and a small number of specific comments.
1.As mentioned briefly in the discussion the study previously by the authors Beuchat et al., 2010a already demonstrated that a deletion in the linker between the BRX-domains could confer increase root vigor. It might be useful to introduce this in slightly more detail in the introduction."

Yes, we now introduce natural allelic BRX variation in more detail (line 53).

"2.Given how much of the study requires comparison between gene structure especially of the linker sequences between species I think it might be useful to include a text figure with schematics of the genes and potentially alignments of the linker sequences. This information is included in supplementary information but may help orientate readers if it is included in the text too."

Indeed, for easier reference we have now added the linker alignment of the genes monitored in our study as a new Figure 1A.

"3.From the evolutionary analysis I think including fern sequences in the alignment would be useful. It was slightly unusual to omit them given the extent of sampling in other lineages."

The reviewer brings up an interesting point! Please note that from the sequences that we retrieved from our search of the OneKP dataset, we limited our alignment (Dataset S1) to bona fide BRX family proteins (i.e. proteins containing the tandem BRX domains and the conserved C-terminal domains). Strikingly, none were found in the ca. 80 fern species sequenced in the OneKP project. However, BLAST searches at NCBI identify a few clear BRX family genes in a few fern species. This could of course also reflect sampling bias. We now point this out in the discussion (line 289).

"4.Given the authors conclusions about the importance of linker length it might have been good to investigate if there are any trends in linker length in different groups of land plants based on the main sequence alignment they carried out. "

Yes, this is of course a good suggestion. At first glance, we cannot see any correlation between linker size and phylogenetic position. Unfortunately, we do not have the competence to perform a more comprehensive phylogenetic analyses and would prefer to leave this to experts in the field. We now discuss the usefulness of such an approach however (line 291).

"5.MpBRXL1 has a short linker sequence but doesn’t increase root vigor. Does this help you narrow down which portion of the linker sequence is important for root vigor rather than simply the length of it – this might be something to mention in the discussion?"

Following up on the point made above, indeed, a phylogenetic analyses combined with experimental verification might give the answer, for example whether there is a size threshold or some inhibitory motif? For now, we point this out in the discussion (line 291).

"Specific comments
Line 26: “non-vascular bryophytes and non-seed lycophytes” what about ferns"

Good point, we have now replaced “angiosperms” with “euphyllophytes” (line 26).

"Line 41: “culminated in the angiosperms” that is a very angiosperm centric view and also makes it sound like evolution has now just reached its end point."

Yes, this was too strong, we have now replaced “culminated in” with “led to” (line 42).

"Line 43: “separates lycophytes”, it separates not vascular plants not just lycophytes."

Yes, but our intention here was a walk along the phylogeny to highlight the groups from which example genes were chosen later on.

"Line 44: “simpler bryophytes” remove the word simpler – does the absence of vascular tissue make bryophytes simple?"

We removed “simpler” (line 45).

"Line 44: “from lycophytes”, within euphyllophytes it separates spermatophytes from ferns not lycophytes."

Yes, indeed, we replaced “lycophytes” with “ferns” (line 45).

"Line 50: “green lineage” it might be good to state this group explicitly "

It is not clear to us what the reviewer means by that?

"Line 104: “basal angiosperm” early diverging lineage of angiosperms"

Yes, we replaced “basal” with “early diverging” (line 112).

Second decision letter

MS ID#: DEVELOP/2022/200917

MS TITLE: Heterologous expression of a lycophyte protein enhances angiosperm seedling vigor

AUTHORS: Samuel W.H. Koh, H. Nicholay Diaz-Ardila, Carlisle Bascom, Eduardo Berenguer, Gwyneth Ingram, Mark Estelle, and Christian Hardtke
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

The manuscript by Koh et al. further develops the study of sub-functionalization of the BRX family proteins that was reported earlier by the authors. In this work heterologous expression of various

BRX genes from different phylogenetic groups (including non-seed plants) was used to evaluated their effect in Arabidopsis. By using a lycophyte shorter version of BRX missing the regulatory phosphosite and with shorter linkers between the BRX domains, the authors observed a dominant, growth-promoting effect in Arabidopsis. It is argued that such BRX variants could serve as biotechnological tools to promote plant growth.
In general, the manuscript is sound and illustrates nicely how such a heterologous expression strategy can learn more about the true nature or original function of protein families.

Comments for the author

I appreciate the effort of the authors to respond and take into account the questions raised by the reviewers on the previous version of this manuscript.
I am satisfied with the changes and have no more questions.
One small remark: I don't like using and making reference to unpublished/peer reviewed studies. The authors make use of a reference to a study of BRX in stomata development by referring to a bioarchives manuscript of 2019:
Rowe, M. H., Dong, J., Weimer, A. K. and Bergmann, D. C. (2019). A Plant-Specific Polarity Module Establishes Cell Fate Asymmetry in the Arabidopsis Stomatal Lineage. bioRxiv.
I don't know the policy of Development in this, but I would argue not to make use of it. After all, it concerns a manuscript that is already 3 years old and not yet published, indicating there is something fundamentally wrong with it.