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CESTA, a positive regulator of brassinosteroid biosynthesis

Brigitte Poppenberger, Wilfried Rozhon, Mamoona Khan, Sigrid Husar, Gerhard Adam, Christian Luschnig, Shozo Fujioka and Tobias Sieberer

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 22 February 2010

Thank you for submitting your manuscript for consideration at The EMBO Journal. It has now been seen by three referees, whose comments are shown below. All of the referees find the study to be in principle interesting, but they require significant further experimental analysis before it can be further considered at the EMBO Journal.

The referee's criticisms focus on the gene expression studies and identification of targets for CESTA. A major criticism raised by all the referees is that the expression studies must be quantitated, this also includes the ChIP experiments. Both referee #2 and #3 recommend that the identification of CESTA targets is extended; importantly, referee #3 requests transcriptome analysis upon CESTA over-expression, and some direct targets validated by ChIP. I believe that these additional experiments would significantly strengthen the study and a suitably revised manuscript would make a good contribution to the EMBO Journal. Should you be able to address these issues experimentally, we would be wiling to re-review a revised manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your

revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This is a very interesting manuscript that reports the discovery and functional study of an Arabidopsis basic helix-loop-helix protein CES. The CES gene was discovered as an activation-tagged gene from an Arabidopsis mutant ces-D, which is morphologically similar to mutants/transgenic plants over-accumulating brassinosteroids (BRs) or exhibiting constitutive BR signaling responses. Interestingly, CES is highly similar to three BEES that were previously known as early BR-response genes and is closely clustered with BEE1 and BEE3 on a phylogenic tree. Using the activation-tagged ces-D, a T-DNA insertional ces-1 null mutant, and several CES transgenic plants, the authors showed that CES is likely required for expression of several Arabidopsis genes (DWF4, CPD, and ROT3) encoding key catalytic enzymes of BR biosynthesis. They also performed experiments to show that CES was somehow regulated post-translationally by BR and could be phosphorylated by BIN2 (a GSK3-like kinase that negatively regulates BR signaling), and that CES interacts with BEE1 and binds G-box elements of the CPD promoter. The manuscript is well written and the data were nice organized. The significance of this study is elucidation of the physiological function of a BEE-like bHLH protein in regulating BR homeostasis.

I have several specific comments about the manuscript:

1. The authors should replace all their semi-quantitative RT-PCR results with quantitative real-time RT-PCR data. They should also examine the transcript levels of DWF4, CPD, and ROT3 in CES-SRDX transgenic lines after 24 h treatment with Brz2001 to see if Brz2001-induced upregulation of the three Arabidopsis genes is significantly inhibited.

2. The 35Spro-CES-SRDX transgenic results are quite nice. However, the dwarf phenotype might be caused by nonspecific effect of extopically-expressed CES-SRDX protein. The authors should cross the ces-1 mutant into the previously described bee1 bee2 bee3 triple mutant to see if simultaneous elimination of 4 bHLH proteins might lead to a dwarf phenotype that can be rescued by exogenous BR application.

3. The fact that nuclear redistribution of CES in response to chemical treatment was only observed in protoplasts but not in cells of stable transgenic plants (mentioned on page 16) suggested that the observed pattern change might be an artifact of the protoplast system due to high abundance of CES proteins. The authors might want to test if these chemical treatments might enhance CES1 binding to the CPD promoter using their ChIP assay.

4. The authors should test if reduced hypocotyl length of the ces-1 mutant is also true for dark-grown seedlings. I was also troubled by attenuated inhibitory effect of Brz2001 on hypocotyl elongation of ces-1 mutant compared to that of the corresponding wild-type seedlings. Based on their hypothesis, one would expect that the ces-1 mutant should be more sensitive to the BR biosynthesis inhibitor.

5. It is well known that dimerization between bHLH proteins can enhance DNA binding activity (Yin et al., Cell 2005). The authors should test if CES-BEE1 interaction could stimulate the CES binding activity to the CPD promoter using the mobility shift assay.

6. The authors showed that CES interacted with both BEE1 and BEE3 in yeast cells but interacted with BEE1 only in protoplasts. Any reasonable explanation for the discrepancy?

7. The authors' claim "CES does not contain a classical GSK3 S/TxxxS/T consensus motif" is not correct. My brief reading of the At1g25330 revealed at least three such sites: STPSS, TDSHS, and SMKLS.

Referee #2 (Remarks to the Author):

In the manuscript, the authors identified a transcription factor, CESTA (CES), this likely functions to promote the expression of brassinosteroid (BR) biosynthesis genes. This is a potentially important finding since not much is known about how BR biosynthesis is positively regulated. Both gain-of-function and loss-of-function mutants are well characterized. However, there is a major need to perform the gene expression studies by more quantitative approach to prove that CES is indeed involved in BR biosynthesis. Several other places need to be strengthened as well.

1. The increase of BR biosynthesis genes in ces-D mutant (Fig. 3C) need to be confirmed by Northern blots or at least quantitative PCR. In addition, the authors should explain why CS and 6-deoxoCS are increased but upstream components are decreased in the BR biosynthesis pathway? Does this indicate that the reaction that converts 6-DeoxoTY (or TY) to DeoxoCS (or CS) is increased in the ces-D mutant? Therefore, it is very important to establish that upstream BR biosynthesis genes are indeed up-regulated.

2. Similarly, the gene expression studies in Fig. 4C also need to be more quantitative. The epi-BL treat experiment need to be carried out with higher concentration (1 to 10 uM), since epi-BL is much less active than brassinolide. The current results in Fig 4C are quite inconsistent and confusing and need to be clarified.

3. The loss-of-function mutant phenotype appears to be very strong in Fig 5; but some of the phenotypes should be quantified. Again, the expression of BR biosynthesis genes need to be quantified, although it is more clear than the studies discussed in 2.

4. The interactions between CES and BEEs are quite convincing. The phosphorylation of CES by BIN2 is rather preliminary and lacks in vivo support.

5.The In vitro binding of CES to G-box is OK. The ChIP studies should be performed by quantitative PCR and, preferably, extend to other BR biosynthesis genes as well.

Referee #3 (Remarks to the Author):

In this manuscript, Poppenberger et al. report on the Identification and functional analysis of CESTA, a bHLH transcription factor involved in signalling of brassinosteroids, a key type of phytohormones. In particular, the authors identified CESTA in an activation tagging population of A. thaliana T-DNA insertional mutants and consistent with this the allele identified is dominant. Then they characterised CESTA expression using Promoter:GUS fusion constructs and study CESTA localisation using GFP translational fusions revealing vasculature as de predominant site of CESTA expression and a clear effect of BRs on CESTA nuclear localisation. The study also includes an analysis of a loss of function cesta mutant displaying weak phenotypic differences with wild type, and , aiming to overcome functional redundancy, of a transgenic plant expressing CESTA fused to the EAR repressor domain, which display stronger phenotypic alterations. The authors also show the effect of altering CESTA activity on Brassinosteroid biosynthesis and the capacity of BR to rescue CESTA:ERA expressing plants. Moreover, using CHIP, the authors show that CESTA binds to the promoter of selected BR biosynthetic genes Finally, CESTA is shown to interact with to BR induced bHLH TF, BEE1 and 2, in Y2H assays and is found to be a, in vitro substrate for BIN2, a kinase in BR signalling pathway. All these data leads the authors to propose that CESTA is involved in the control of BR biosynthesis. In this reviewers opinion this is a quite complete study

which provides strong data positioning CSETA in BR signalling. However, the conclusion that CESTA key role is on the control of BR biosynthesis is not sufficiently demonstrated. Yes, CESTA repression is alleviated by BR addition, and CESTA controls BR biosynthetic genes, but given the existence of feedback regulatory loops, their conclusion is not sufficiently sustained. An analysis of CESTA overexpresion on a BR biosynthesis mutant background or a transcriptomic analysis in in CESTA- and CESTA:EAR overexpresor lines, together with CHIP examination of a couple of G-box containing potential targets, would be required to hold their statement.

In summary, this reviewer finds the study important and of interest as it contributes to the unveiling of a key phytohormone signalling pathway, but requires additional substantiation of their key main claim.

Minor point

The authors study CESTA expression based on promoter GUS fusions. Given that it is known that some cis-regulatory regions can resite outside the promoter, the authors should provide additional confirmation of their findings, at least for some organs/plant parts examined. Comparing their data with that in GENEVESTIGATOR would be sufficient

1st Revision - authors' response 22 May 2010

We thank the reviewers for their comments on our manuscript (tracking no. #EMBOJ-2010-73680). The specific points raised by the reviewers were addressed as follows:

Reviewer 1:

This is a very interesting manuscript that reports the discovery and functional study of an Arabidopsis basic helix-loop-helix protein CES. The CES gene was discovered as an activation-tagged gene from an Arabidopsis mutant ces-D, which is morphologically similar to mutants/transgenic plants over-accumulating brassinosteroids (BRs) or exhibiting constitutive BR signaling responses. Interestingly, CES is highly similar to three BEES that were previously known as early BR-response genes and is closely clustered with BEE1 and BEE3 on a phylogenic tree. Using the activation-tagged ces-D, a T-DNA insertional ces-1 null mutant, and several CES transgenic plants, the authors showed that CES is likely required for expression of several Arabidopsis genes (DWF4, CPD, and ROT3) encoding key catalytic enzymes of BR biosynthesis. They also performed experiments to show that CES was somehow regulated post-translationally by BR and could be phosphorylated by BIN2 (a GSK3-like kinase that negatively regulates BR signaling), and that CES interacts with BEE1 and binds G-box elements of the CPD promoter. The manuscript is well written and the data were nice organized. The significance of this study is elucidation of the physiological function of a BEE-like bHLH protein in regulating BR homeostasis.

We are delighted that this reviewer finds our work very interesting and of significance!

I have several specific comments about the manuscript:

1. The authors should replace all their semi-quantitative RT-PCR results with quantitative real-time RT-PCR data. They should also examine the transcript levels of DWF4, CPD, and ROT3 in CES-SRDX transgenic lines after 24 h treatment with Brz2001 to see if Brz2001-induced upregulation of the three Arabidopsis genes is significantly inhibited.

To answer the concern of this reviewer we have repeated the expression analyses and have quantified transcript levels using quantitative real-time PCR. We have either replaced the semi-

quantitative RT-PCR data with the quantitative results or, in one instance, have provided the qPCR results in addition.

In regard to the CES-SRDX plants the expression analysis using qPCRs and the transcriptome analysis have now shown quantitatively that, as opposed to a clear upregulation of DWF4, CPD and ROT3 in ces-D plants, the reduction in CPD and ROT3 mRNA levels in 35Sp:cMyc-CES-SRDX plants has to be considered as very subtle. DWF4 expression seems unaffected, if not even slightly induced. This result may be explained by the fact that, according to the microarray data, CES expression in the dominant ces-D mutant is approximately six times more increased than CES-SRDX expression in 35Sp:cMyc-CES-SRDX plants.

We show in our work that ces-1 exhibits some degree of attenuation of induction of BR biosynthesis genes in response to Brz2001. Thus, also in light of the next comment of this reviewer, that effects seen in CES-SRDX plants may be caused by ectopic expression of CES-SRDX, we decided not to repeat these response assays also with CES-SRDX plants.

2. The 35Spro-CES-SRDX transgenic results are quite nice. However, the dwarf phenotype might be caused by nonspecific effect of extopically-expressed CES-SRDX protein. The authors should cross the ces-1 mutant into the previously described bee1 bee2 bee3 triple mutant to see if simultaneous elimination of 4 bHLH proteins might lead to a dwarf phenotype that can be rescued by exogenous BR application.

This is clearly an important experiment to be conducted and we are currently in the process of trying to obtain a ces-1 bee1 bee2 bee3 mutant. However it is a very time-consuming process to generate a quadruple mutant and we thus consider it out of the scope of this study to generate such a mutant line.

3. The fact that nuclear redistribution of CES in response to chemical treatment was only observed in protoplasts but not in cells of stable transgenic plants (mentioned on page 16) suggested that the observed pattern change might be an artifact of the protoplast system due to high abundance of CES proteins. The authors might want to test if these chemical treatments might enhance CES1 binding to the CPD promoter using their ChIP assay.

This is a very important point, which we have now addressed in detail. Clearly the lack of nuclear redistribution in response to BL treatment in plants stably transformed with a CES-YFP reporter raised concerns that BR-induced compartmentalization of CES-YFP in nuclei of protoplasts may be an artefact. We have thus conducted a detailed investigation of CES-YFP expression in Arabidopsis in response to treatments with BL or a BR biosynthesis inhibitor, to examine the effects of alterations in BR signaling on reporter localization. In these experiments we confirmed that a two-hour BL treatment was not sufficient to induce CES-YFP nuclear compartmentalization in planta. However, when plants were pre-treated with a BR biosynthesis inhibitor for 24 hours, a two-hour BL treatment induced CES-YFP re-localization to subnuclear compartments. Thus interestingly it appears that CES-YFP changes its subnuclear localization specifically in response to a rapid induction of BR signaling in planta. We have included this new result in the manuscript.

4. The authors should test if reduced hypocotyl length of the ces-1 mutant is also true for dark-grown seedlings. I was also troubled by attenuated inhibitory effect of Brz2001 on hypocotyl elongation of ces-1 mutant compared to that of the corresponding wild-type seedlings. Based on their hypothesis, one would expect that the ces-1 mutant should be more sensitive to the BR biosynthesis inhibitor.

CES misexpressing plants show light-dependent phenotypes, which will be published as part of a separate study.

It is difficult to predict which growth response is to be expected of ces-1 following Brz2001 application, as the role of CES in BR signaling is not as yet well defined. However at the molecular level ces-1 shows an attenuated response of BR biosynthetic gene expression to BL and Brz2001 application. Thus we consider that this attenuated adjustment of BR biosynthesis may also impact on the adaptation of growth responses in response to Brz2001 application.

5. It is well known that dimerization between bHLH proteins can enhance DNA binding activity (Yin et al., Cell 2005). The authors should test if CES-BEE1 interaction could stimulate the CES binding activity to the CPD promoter using the mobility shift assay.

We agree with this reviewer that this is an interesting experiment to be performed and thus we have aimed to analyse, if a presence of BEE1 would alter CES DNA binding in vitro. However, although it seamed straight forward, the experiment turned out to be a challenging one, as we were unable to obtain recombinant BEE1 protein. As opposed to CES BEE1 was insoluble in E. coli and, though several attempts were made and different systems were used, we did not succeed in expressing BEE1 within the time given for this revision. Thus very unfortunately we cannot provide this result at present.

6. The authors showed that CES interacted with both BEE1 and BEE3 in yeast cells but interacted with BEE1 only in protoplasts. Any reasonable explanation for the discrepancy?

The result that, in our hands, CES did not appear to interact with BEE3 in spilt-YFP assays is difficult to interpret. In principle it is conceivable that CES does not interact with BEE3 in vivo. However we consider it more likely that the reporter did not reconstitute due to technical problems. For example BEE3 expression is lower in protoplasts than that of BEE1, which may impair reconstitution of sufficient amounts of YFP to be detected.

7. The authors' claim "CES does not contain a classical GSK3 S/TxxxS/T consensus motif" is not correct. My brief reading of the At1g25330 revealed at least three such sites: STPSS, TDSHS, and SMKLS.

Thank you for pointing out that this was a wrong statement: we forgot to mention that classical GSK3 consensus motifs are S/TxxxS/T sequences in tandem repeats (which is a motif not present in CES). We have now corrected this in the discussion.

Reviewer 2:

In the manuscript, the authors identified a transcription factor, CESTA (CES), this likely functions to promote the expression of brassinosteroid (BR) biosynthesis genes. This is a potentially important finding since not much is known about how BR biosynthesis is positively regulated. Both gain-of-function and loss-of-function mutants are well characterized. However, there is a major need to perform the gene expression studies by more quantitative approach to prove that CES is indeed involved in BR biosynthesis. Several other places need to be strengthened as well.

We are happy that this reviewer considers our findings of potentially important and have addressed his/her concerns/remarks as follows:

1. The increase of BR biosynthesis genes in ces-D mutant (Fig. 3C) need to be confirmed by Northern blots or at least quantitative PCR. In addition, the authors should explain why CS and 6-deoxoCS are increased but upstream components are decreased in the BR biosynthesis pathway? Does this indicate that the reaction that converts 6-DeoxoTY (or TY) to DeoxoCS (or CS) is increased in the ces-D mutant? Therefore, it is very important to establish that upstream BR biosynthesis genes are indeed up-regulated.

As requested we have conducted qPCR analysis of BR biosynthetic gene expression in ces-D as compared to wild type, which has confirmed our previous semi-quantitative analysis. We present both sets of independent biological replicates in the manuscript (Fig. 3c semi-quantitative data, Fig. 5c qPCR data).

As to the second point, apparently the detailed discussion of the BR profiling results has been overlooked: indeed it is interesting that earlier intermediates of BR biosynthesis, namely 6-DeoxoTY and TY levels were decreased in ces-D. This suggests that CES regulates (a) gene(s) essential for BR C-2 hydroxylation, which (is) are as yet unidentified in Arabidopsis (see also discussion).

2. Similarly, the gene expression studies in Fig. 4C also need to be more quantitative. The epi-BL treat experiment need to be carried out with higher concentration (1 to 10 uM), since epi-BL is much less active than brassinolide. The current results in Fig 4C are quite inconsistent and confusing and need to be clarified.

We have repeated the gene expression studies in ces-1 as compared to wild-type and have replaced the original data with the new results. The results show that in ces-1 DWF4 and ROT3 expression is markedly reduced as opposed to wild-type. However CPD mRNA levels were not altered in ces-1 plants. It seams possible that functional redundancy in the regulation of CPD expression accounts for this lack of reduction in CPD transcript levels.
As we did have a strong down-regulation of DWF4, CPD and ROT3 expression in response to the amount of epiBL used, we do not consider it necessary to increase this concentration, especially also since physiological BL concentration are in the pM to nM range only.
We hope that the way in which we have now presented the quantitative results of changes in gene expression in Figure 4C will be easier to read.

3. The loss-of-function mutant phenotype appears to be very strong in Fig 5; but some of the phenotypes should be quantified. Again, the expression of BR biosynthesis genes need to be quantified, although it is more clear than the studies discussed in 2.

In response to the concern of this reviewer we have chosen to measure petiole length in seedlings of 35Sp:cMyc-CES-SRDX plants and wild-type and present this data quantitatively in Fig. 5. The changes are significant, with the strength of phenotype correlating with the amount of recombinant protein present in the independent transgenic lines.

Moreover, as requested, we have also quantified the expression of CPD, ROT3 and DWF4 in 35Sp:cMyc-CES-SRDX plants using qPCRs. A discussion of the results obtained in this analysis is provided earlier in this letter (response to point 1 of reviewer 1).

4. The interactions between CES and BEEs are quite convincing. The phosphorylation of CES by BIN2 is rather preliminary and lacks in vivo support.

We agree with this reviewer that in light of the results we present in this work it is too preliminary to speculate about the possibility that BIN2 directly controls CES action by phosphorylation in vivo. However the fact that CES-YFP re-localizes in response to treatment with Bkn, an inhibitor of BIN2 action, indicates that BIN2 may impact on CES action and/or localization either directly or indirectly. We are currently in the process of investigating the possibility that BIN2 directly regulates CES action by phosphorylation in vivo and are investigating a potential physiological significance of such phosphorylation events.

5.The In vitro binding of CES to G-box is OK. The ChIP studies should be performed by quantitative PCR and, preferably, extend to other BR biosynthesis genes as well.

We have now quantified the ChIP experiments analysing CES binding to the CPD promoter using qPCRs and included this data in the manuscript.

We fully agree with reviewer 2 that the identification of additional CES targets is of high interest, however as outlined also in the response to reviewer 3 later in this letter, it is a very challenging and time-consuming task and we thus consider it as scope of a follow-up study.

Reviewer 3:

In this manuscript, Poppenberger et al. report on the Identification and functional analysis of CESTA, a bHLH transcription factor involved in signalling of brassinosteroids, a key type of phytohormones. In particular, the authors identified CESTA in an activation tagging population of A. thaliana T-DNA insertional mutants and consistent with this the allele identified is dominant. Then they characterised CESTA expression using Promoter:GUS fusion constructs and study CESTA localisation using GFP translational fusions revealing vasculature as de predominant site of CESTA expression and a clear effect of BRs on CESTA nuclear localisation. The study also includes

an analysis of a loss of function cesta mutant displaying weak phenotypic differences with wild type, and , aiming to overcome functional redundancy, of a transgenic plant expressing CESTA fused to the EAR repressor domain, which display stronger phenotypic alterations. The authors also show the effect of altering CESTA activity on Brassinosteroid biosynthesis and the capacity of BR to rescue CESTA:ERA expressing plants. Moreover, using CHIP, the authors show that CESTA binds to the promoter of selected BR biosynthetic genes Finally, CESTA is shown to interact with to BR induced bHLH TF, BEE1 and 2, in Y2H assays and is found to be a, in vitro substrate for BIN2, a kinase in BR signalling pathway. All these data leads the authors to propose that CESTA is involved in the control of BR biosynthesis. In this reviewers opinion this is a quite complete study which provides strong data positioning CSETA in BR signalling.

We are happy that this reviewer considers our study to be complete and concludes that we provide strong data to position CES in BR signaling.

However, the conclusion that CESTA key role is on the control of BR biosynthesis is not sufficiently demonstrated. Yes, CESTA repression is alleviated by BR addition, and CESTA controls BR biosynthetic genes, but given the existence of feedback regulatory loops, their conclusion is not sufficiently sustained. An analysis of CESTA overexpresion on a BR biosynthesis mutant background or a transcriptomic analysis in in CESTA- and CESTA:EAR overexpresor lines, together with CHIP examination of a couple of G-box containing potential targets, would be required to hold their statement.

We have addressed the concern of this reviewer by performing the required transcriptome analysis of gene expression in ces-D and the 35Sp:cMyc-CES-SRDX plants. The results of these microarrays are presented as a summary in Table 1 and are also included in full in the supplementary material.
As to the second point the identification of additional CES targets is clearly of high interest. However we did not consider transcriptome data of ectopically expressing CES and CES-SRDX plants as being potentially useful in the identification of putative direct CES targets. We were concerned that secondary effects on gene expression would mask primary responses caused by elevated CES expression. This is why we are in the process of constructing plant lines in which CES and CES-SRDX expression can be transiently induced. We will use these plants to analyse transcriptome changes at different time-points after induction and hope that an early time-point will yield genes, which CES directly regulates and thus can bind to.
The results of the transcriptome analysis of ces-D and CES-SRDX plants have now confirmed our concern, as a large number of genes strongly up-regulated in ces-D code for transcription factors and other transcriptional regulators (whose activation will likely result in changes in expression of their targets). Nevertheless, in the very limited amount of time, which had remained after we had received the microarray data, we did analyze CES binding to the promoters of two genes namely PHE2 and DIN11, which are both strongly upregulated in ces-D and also contain G-boxes. Unfortunately CES bound to neither of both.

In summary we consider the identification of additional CES targets as very important, but also as a very challenging and time-consuming task and thus as scope of a follow-up study.

Minor point

The authos study CESTA expression based on promoter GUS fusions. Given that it is known that some cis-regulatory regions can resite outside the promoter, the authors should provide additional confirmation of their findings, at least for some organs/plant parts examined. Comparing their data with that in GENEVESTIGATOR would be sufficient.

It seems that it has been over-looked that we have done such a comparison to validate our GUS reporter results, which was also mentioned in the result section.

2nd Editorial Decision 29 June 2010

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. It has now been seen by the three original referees whose comments are shown below. As you will see from their comments two of the referees find that a couple of concerns remain that currently preclude publication in The EMBO Journal.

The major issues that remain surround the transcriptomic data provided in the revised manuscript, this includes the lack of a link between Cesta and BR biosynthetic enzymes together with the inconsistencies with the steroid profiling data as raised by referee #1. In addition this referee requires the genetic support for the functional link between CES-BEE. Referee #3 also raises concerns with the transcriptomic data, the way it is presented and importantly, inconsistencies between the CES and CES-SRDX data, this clearly needs to be addressed, again suggesting the use of the quadruple mutant. I have also noted that you have not been able to ChIP CES to target genes, this is also a concern and must be addressed. It is the EMBO Journal policy to allow one round of revision, however, the referees appreciate that you have put in significant effort to address the previous comments and if you are able to satisfactorily address these remaining concerns we would consider a second and final revised manuscript.

When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

After analyzing the microarray data and the new quantitative RT-PCR results, I doubt that the phenotypes observed with the ces-D or CES-SRDX are caused by altered expression of the CPD (2.38 fold increase in ces-D and 0.70 fold reduction in CES-SRDX) or ROT3 gene. One of the highly induced genes is At1g48800 encoding a terpene synthase/cyclase that might be involved in terpenoid biosynthesis. Besides, the steroid profiling data indicated that the most likely steps (C-2 hydroxylation) affected by the ces-D mutation are not catalyzed by CPD, DWF4, or ROT3. The DWF4 catalyzes the C22-hydroxylation reaction; ROT3 functions redundantly with CYP740D1 to catalyze the C23-hydroxylation while CPD was thought to catalyze a step upstream of the C23-hydroxylation. Thus, I don't think that the evidence presented in this manuscript provides a strong support for the CESTA-CPD-Brassinosteroid model.

The authors complained that it is a very time-consuming process to generate a ces bee1 bee2 bee3 quadruple mutant, but they could use the RNA-interference or artificial microRNA method to silence the CESTA gene in the bee triple mutant to convincingly show its redundant role in brassinosteroid biosynthesis. This should provide a crucial genetic support for the CES-BEE physical interaction.

Referee #2 (Remarks to the Author):

The authors largely addressed my concerns. The authors should write more clearly about microarray data analysis. First, the description of "P-value" seems problematic. In general, the bigger P-value, the less significant the data is. If the "current P-value" is (1-"real P-value"), then it makes sense. The statistical methods (like software, algorithm etc.) used in the analysis should be provided. Finally it would be nice if the authors can compare the CES1 affected genes with published BR-regulated genes to see how much overlaps there are.

Referee #3 (Remarks to the Author):

In this new version of the manuscript by Poppenberger et al. the authors have made an important effort to respond to reviewers criticisms. In general, all new results have clearly improved the quality of the new version, with the notable exception of transcriptomic data. Indeed, there are various aspects of the data, which need reconsideration. One is the presentation of the data. In its present format it is somewhat poor; for instance the authors use the p value as a test for quality of the data (actually it is more common to use the 1-p value for probability that the differences in the samples being compared is due to chance). It is now accepted that FDR (False discovery rate) is a better estimator). In addition, no reference to this data is made on the discussion section and the table in the text is not much informative. Diagrams showing overlaps (genes induced or repressed in both CES and CES-SRDX versus wild type) and antioverlaps (genes induced in CES and repressed in CES-SRDX and the reciprocal) are needed to understand the results. Second, and more important are the results per se. A brief analysis of the data in the corresponding supplementary table reveals that, contrary to simple expectations, there are a higher number of genes that display the same behavior in both CES and CES-SRDX than those that display the opposite behavior. It is reasonable to think that this finding suggests that CES1 per se is also a repressor of some genes, or that the activator function of CES in CES-SRDX has not been fully impaired, or also that regulatory loops are operating. In any case, this data raises questions on the validity of other data with CES-SRDX transgenic plants, thus questioning acceptability for publication of the manuscript in the present formulation. Potential ways to overcome these problems are i) to replace CES-SRDX data with data obtained with a double, triple or quadruple mutant in which CES and potential functional homologs are impaired; or ii) to examine the transcriptome of CES and CES-SRDX after brassinosteroid mutant. In case results are coherent, the suitability for publication of the new version of the manuscript could be reconsidered

Minor point
Again, given the data presented, this reviewer favors that the conclusion that CES1 exclusively controls brassinosteroid biosynthetic genes is boiled down. For instance, "CES1 is involved in brassinosteroid signaling, at least in part by controlling brassinosteroid biosynthesis".

2nd Revision - authors' response 27 October 2010

We thank the reviewers for their comments on our manuscript (tracking no. #EMBOJ-2010-73680R) and would like to address the specific points raised as follows:

Reviewer 1:

After analyzing the microarray data and the new quantitative RT-PCR results, I doubt that the phenotypes observed with the ces-D or CES-SRDX are caused by altered expression of the CPD (2.38 fold increase in ces-D and 0.70 fold reduction in CES-SRDX) or ROT3 gene. One of the highly induced genes is At1g48800 encoding a terpene synthase/cyclase that might be involved in terpenoid biosynthesis. Besides, the steroid profiling data indicated that the most likely steps (C-2 hydroxylation) affected by the ces-D mutation are not catalyzed by CPD, DWF4, or ROT3. The DWF4 catalyzes the C22-hydroxylation reaction; ROT3 functions redundantly with CYP740D1 to

catalyze the C23-hydroxylation while CPD was thought to catalyze a step upstream of the C23-hydroxylation. Thus, I don't think that the evidence presented in this manuscript provides a strong support for the CESTA-CPD-Brassinosteroid model.

Indeed we completely agree with this reviewer and have already discussed in the last version of our manuscript, that the BR profile of ces-D indicates that ces-D up-regulates the C-2 hydroxylation step in BR biosynthesis. We have also stated that the cytochrome P450 catalyzing this step, and thus the gene coding for it, is presently unknown in Arabidopsis. Interestingly, when we performed further ChIP experiments (requested by the editor), we identified another CES target gene: CYP718. CYP718 is the cytochrome P450 most strongly induced in ces-D and encodes a close homologue of the CYPs 85A1 and 85A2, P450 that catalyze BR 6-oxydation. We discuss the possibility that CYP718 may be involved in BR biosynthesis, a hypothesis which we are currently verifying. However, in addition to the fact that ces-D impacts on BR 2-hydroxylation (evidence by the BR profile of the mutant), our results also show, that ces-D has effects on the expression of known BR biosynthesis genes. BR biosynthetic genes are strongly feedback suppressed in their transcription by BRs. As discussed: 'the fact that in ces-D, in spite of increased levels of 6-DeoxoCS and CS, feedback control did not set in, but on the contrary DWF4, CPD and ROT3 were induced, suggested that CES also impacts on the regulation of these genes.' Our qPCR results show that this induction of DWF4, CPD and ROT3 in ces-D is at least two-fold, which is a magnitude of regulation characteristic for BR-regulated genes. We present different pieces of evidence, which show that CES participates in the regulation of DWF4, CPD and ROT3 expression; whether CES plays a primary (direct) role in these regulatory events is as yet not fully resolved. We do show that in principle CES can bind to the promoter of CPD in planta, suggesting that (one) physiological role of CES may be to impact on the CPD mRNA abundance.

To address the concerns of this reviewer, we have modified parts of the msc aiming at presenting and explaining our data and conclusions more clearly.

The authors complained that it is a very time-consuming process to generate a ces bee1 bee2 bee3 quadruple mutant, but they could use the RNA-interference or artificial microRNA method to silence the CESTA gene in the bee triple mutant to convincingly show its redundant role in brassinosteroid biosynthesis. This should provide a crucial genetic support for the CES-BEE physical interaction.

This reviewer suggests the application of obvious strategies for the generation of knock-down plants, and we have applied both RNAi and the use of artificial microRNAs, aiming at reducing the expression of BEE1 and BEE3 (the closest homologues of CES), in the ces-1 knock-out background. However neither approach yielded plants in which the expression of all three genes was efficiently reduced. Thus we have crossed the bee1,2,3 triple mutant with ces-1, to obtain a quadruple knock-out mutant. As we have explained previously this is time-consuming (according to mendelian genetics approx. 750 F2 progeny will have to be obtained and screened by genotyping the four genes). Thus we consider this out of the scope of this revision.

Reviewer 2:

The authors largely addressed my concerns. The authors should write more clearly about microarray data analysis. First, the description of "P-value" seems problematic. In general, the bigger P-value, the less significant the data is. If the "current P-value" is (1-"real P-value"), then it makes sense. The statistical methods (like software, algorithm etc.) used in the analysis should be provided. Finally it would be nice if the authors can compare the CES1 affected genes with published BR-regulated genes to see how much overlaps there are.

We are glad that we have largely addressed the concerns of this reviewer! We have now changed the P-values to the appropriate and requested values. To investigate, if BR induced genes are over-represented among ces-D up-regulated genes, we have conducted a statistical analysis. This analysis has revealed that BR-induced genes are highly significantly enriched among genes up-regulated by ces-D. A table listing these genes was compiled (Supp Table 2) and, in addition to a discussion of this new data, included in the manuscript.

Reviewer 3:

In this new version of the manuscript by Poppenberger et al. the authors have made an important effort to respond to reviewers criticisms. In general, all new results have clearly improved the quality of the new version, with the notable exception of transcriptomic data. Indeed, there are various aspects of the data, which need reconsideration. One is the presentation of the data. In its present format it is somewhat poor; for instance the authors use the p value as a test for quality of the data (actually it is more common to use the 1-p value for probability that the differences in the samples being compared is due to chance). It is now accepted that FDR (False discovery rate) is a better estimator). In addition, no reference to this data is made on the discussion section and the table in the text is not much informative. Diagrams showing overlaps (genes induced or repressed in both CES and CES-SRDX versus wild type) and antioverlaps (genes induced in CES and repressed in CES-SRDX and the reciprocal) are needed to understand the results.

We thank this reviewer for acknowledging our effort to respond to the critisism of the reviewers and for his/her remark that the additional data have clearly improved the quality of our manuscript. We agree that the presentation of the microarray results was poor in the first revision (due to limited amount of time after having received the raw data). Thus, in response, we have now changed the presentation as requested and as follows:

(1) The P-values were changed accordingly,

(2) diagrams showing over-laps and anti-overlaps were included

(3) an analysis of the presence of G-box motifs in both ces-D and CES-SRDX controlled genes was added and

(4) a statistical analysis of the frequency of BR-induced genes among ces-D up-regulated ones was performed and a table listing these genes was compiled.

This additional evaluation of the data has revealed interesting new results, which were included in the revised version of the manuscript and are also discussed. On the one hand it became apparent that a statistically highly significant number of genes up-regulated in ces-D are known to be BR-induced. On the other hand, an analysis of the presence of G-box motifs in the 5' UTRs of genes miss-regulated in ces-D revealed that G-boxes, which we show are binding sites of CES, are significantly enriched in the promoters of ces-D induced genes, and correspondingly also in genes repressed in CES-SRDX expressing plants.

Second, and more important are the results per se. A brief analysis of the data in the corresponding supplementary table reveals that, contrary to simple expectations, there are a higher number of genes that display the same behavior in both CES and CES-SRDX than those that display the opposite behavior. It is reasonable to think that this finding suggests that CES1 per se is also a repressor of some genes, or that the activator function of CES in CES-SRDX has not been fully impaired, or also that regulatory loops are operating. In any case, this data raises questions on the validity of other data with CES-SRDX transgenic plants, thus questioning acceptability for publication of the manuscript in the present formulation. Potential ways to overcome these problems are i) to replace CES-SRDX data with data obtained with a double, triple or quadruple mutant in which CES and potential functional homologs are impaired; or ii) to examine the transcriptome of CES and CES-SRDX after brassinosteroid mutant. In case results are coherent, the suitability for publication of the new version of the manuscript could be reconsidered.

This is an important point raised and we have included a detailed discussion of the CES-SRDX data in the manuscript to address the concern. However, also in light of the now included thorough evaluation of the microarray results, we do not believe that the fact that the anti-overlap of genes significantly induced in ces-D and genes significantly repressed in 35Sp:CES-SRDX is small (and that genes exist which are induced in both lines), suggests that CES-SRDX is not functional. There

is a high number of transcriptional regulators, which are miss-regulated in CES and CES-SRDX over-expressing plants. These are expected to induce secondary changes in transcriptional networks that may mask CES and CES-SRDX primary effects. This also correlates with the fact that G-boxes are not enriched in ces-D repressed and CES-SRDX induced genes. Moreover it seems likely that CES-SRDX and CES may not have equal transcriptional activities in the lines analysed. For example CES expression in ces-D is approximately 5 times higher, than CES-SRDX expression in the line with highest CES-SRDX mRNA levels (203), which was used for the microarray analysis (Supplementary Table 1). These differences in expression are likely to impact on the extend, by which target genes are regulated. Thus, for example although genes may in fact be repressed by CES-SRDX, they may not be identified as such in 35Spro:c-Myc-CES-SRDX/203 seedlings (and are thus lost to yield an anti-overlap with ces-D induced genes) with the stringent parameters chosen for the statistical analysis.

The result that 35Spro:c-Myc-CES-SRDX/203 plants are characterized by BR deficient growth morphologies, which are reverted to wild-type by external BL application (and thus oppose ces-D phenotypes), argues for CES-SRDX being functional as a repressor of CES targets. And indeed for example CPD, which is significantly induced in ces-D (and to the promoter of which CES can bind to in planta) is also statistically significantly reduced in 35Spro:c-Myc-CES-SRDX/203, albeit to an extend (0.7 fold), which falls below the trash-hold levels set for statistical analysis.

Minor point

Again, given the data presented, this reviewer favors that the conclusion that CES1 exclusively controls brassinosteroid biosynthetic genes is boiled down. For instance, "CES1 is involved in brassinosteroid signaling, at least in part by controlling brassinosteroid biosynthesis".

We don't believe that this suggested title would be appropriate for the manuscript, as we do not provide sufficient evidence to state that CES is involved in BR signalling. We do show in this work that CES can regulate BR biosynthetic gene expression, thus we feel the title we chose is justified. However, to address the concern, we have included a detailed discussion in the manuscript, which points out that our results suggest that CES, in addition to its role in BR biosynthesis, also impacts on the regulation of other BR responses. Also we have tried to make

3rd Editorial Decision 01 December 2010

Your revised manuscript has been reviewed once more by one of the original referees and there are a few minor issues that need to be resolved prior to publication. Pending satisfactory minor revision, we would be willing to consider publishing your manuscript in the EMBO Journal.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #3 (Remarks to the Author):

In this new version of the manuscript, the authors made some elaboration on the transcriptomic data. As a result, they find that i) the gene set displaying increased expression in ces-D is highly enriched in BR-responsive genes, ii) the promoters of this gene set are enriched in G-box motifs. Iii) reciprocally, the promoters of the gene set displaying decreased expression in CES-SRDX are enriched in G-boxes. These findings are fully consistent with a role of CES in BR signalling. In this context, I agree that the finding that several genes display increased or decreased expression in both CES and CES-SRDX, may be due to the intrinsic complexity of the BR signalling network, with positive and negative feedback loops that may obscure the primary effects of altering the activity of BR signalling genes. On the other hand, given the enrichment of G boxes in the promoters of the authors should raise the possibility that, in addition to BR biosynthetic genes, it is likely that CES directly controls other BR responsive genes. A note of caution regards the microarray data per se. It is unclear for this reviewer whether their P value refers to a "corrected P value" or it is just the P value obtained using a Student t-test. In such case, the authors should recalculate the corrected P-value (which estimates the false discovery rate). Additionally, the authors should carefully check for microarray data variation between different replicas, leading to modest P values even when average differences in expression are pretty high. For instance, for At1g61710 expression in CES-SRDX is 13-fold higher than in wild type, yet the P value is .06. This reviewer wonders whether this reflects an anomalous behaviour of one of the three biological replicas (corresponding to one or more of the genotypes analysed, CES-D, CES- SRDX and wt). The authors should check this possibility using an statistic test (eg, Pearson correlation coefficient).

3rd Revision - authors' response 17 December 2010

I thank you and the reviewer for your comments on the second revision of our manuscript (tracking no. #EMBOJ-2010-73680R1) entitled: "CESTA a positive regulator of brassinosteroid biosynthesis" by Brigitte Poppenberger, Wilfried Rozhon, Mamoona Khan, Sigrid Husar, Gerhard Adam, Christian Luschnig, Shozo Fujioka and Tobias Sieberer

The reviewer that has once more reviewed our work acknowledges that the results of the evaluation of the microarray fully support a role of CES in BR signaling. In this context she/he points out that *'given the enrichment of G boxes in the promoters of the authors should raise the possibility that, in addition to BR biosynthetic genes, it is likely that CES directly controls other BR responsive genes'.* We fully agree with this comment and had therefore discussed this possibility previously (pg. 5 and pg. 17).

To respond to this remark we have:

1.) changed the concluding sentence of the Introduction to: 'We discuss a model in which CES is regulated by BIN2 action, to allow for a control of BR biosynthesis and also of other BR responses.' and

2.) changed the last sentence of the abstract from:

Moreover we show that CESTA subnuclear localization is controlled by BRs and discuss a model, in which CESTA interplays with BEE1 to control BR biosynthesis.
to:
Moreover we show that CESTA subnuclear localization is BR-regulated and discuss a model, in which CESTA interplays with BEE1 to control BR biosynthesis and other BR responses.

3.) To keep the word count of the abstract below 175 the third sentence was also changed from:

However, whilst it is recognised...
to: Whilst it is recognised ...

The second point raised by the reviewer concerns the calculated p-values:

A note of caution regards the microarray data per se. It is unclear for this reviewer whether their P value refers to a "corrected P value" or it is just the P value obtained using a Student t-test. In such case, the authors should recalculate the corrected P-value (which estimates the false discovery rate).

To respond to this concern we have now, in addition to the Student's t-test p-values, also calculated FDR adjusted p-values (applying the frequently used cut-off of 0.10) and

4.) have added the resulting q-values to Supplementary Figure 1 of the manuscript. Using the q-values we have re-evaluated our data in regard to over-laps and anti over-laps of ces-D and CESSRDX regulated genes, the number of G-boxes in the promoters of ces-D induced or CES-SRDX repressed genes and the amount of BR-responsive genes among ces-D induced genes. These recalculations yielded results that were only marginally different to the ones previously obtained when using p-values (with a cut-off of 0.05).

5.) The new numbers were included in Figure 6 as well as in the result section on pg. 10 and 11.

Moreover
6.) the Supplementary tables were adjusted to incorporate the q-values.

7.) Also the method of q-value calculation was added to the Materials and methods section.

Additionally, the authors should carefully check for microarray data variation between different replicas, leading to modest P values even when average differences in expression are pretty high. For instance, for At1g61710 expression in CES-SRDX is 13-fold higher than in wild type, yet the P value is .06. This reviewer wonders whether this reflects an anomalous behaviour of one of the three biological replicas (corresponding to one or more of the genotypes analysed, CES-D, CES- SRDX and wt). The authors should check this possibility using an statistic test (eg, Pearson correlation coefficient).

To investigate whether excluding outliers based on statistics, would impact on our results and conclusions, we applied the Dixon's Q-test. As expected, the number of up- and down-regulated genes increased slightly (e.g. from 370 genes up-regulated in ces-D as compared to wild-type, to 400). When the filtered data sets were used for further analyses, the results did not change notably (as compared to those obtained with the unfiltered data sets). For example, the frequency of genes containing G-boxes in the promoters of ces-D induced genes was 0.421 with the unfiltered data set and 0.423 with the filtered data set. However, in the gene expression values obtained for locus At1g61710, which was mentioned by the reviewer, no outlier was identified with Dixon's Q-test. We agree with this reviewer that a proper evaluation of microarray results is crucial. However, it is widely acknowledged that the mathematical identification of outliers is challenging, if the number of replicates is relatively small, as there tends to be a high error rate. Therefore we consider it problematic to apply statistics, aiming at identifying outliers, to our data sets.

8.) To enable readers to evaluate single genes identified as significantly regulated in our work themselves, we have now also included the raw data of the microarray experiments as a Supplementary Table (Supplementary Table 2).

4th Editorial Decision 17 January 2011

Thank you for submitting a final revised version of your manuscript. It has been reviewed once more by one of the original referee (#3) who finds that you have

satisfactorily addressed all the remaining concerns and now recommends publication in the EMBO Journal. I realise that is has taken a lot of additional work since the original submission, but I believe that the final manuscript will make a good contribution to the journal. You will receive the official acceptance letter in the next couple of days.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #3

The authors have properly addressed my concerns on the satistical analyses of microarray data. Moreover, I determined the pearson corrrelation coefficient of the three replicas of each genotype and found they are ca .99, which is pretty good.

