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# Developmental regulation of CYCA2s contributes to tissue-specific proliferation in Arabidopsis

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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 25 February 2011

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see all three referees appreciate the link between cell cycle coordination and differentiation and find the manuscript in principle suitable for the EMBO Journal. They raise different concerns with the study that have to be resolved before further consideration here. Given the comments provided by the referees, I would like to invite you to submit a suitably revised manuscript for our consideration. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that it is therefore important to address the concerns raised at this stage. 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 your revision.

Yours sincerely,

Editor
The EMBO Journal

 

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Vanneste et al. deals in this manuscript with a question of major relevance, understanding the coordination of cell proliferation and differentiation in morphogenesis. The family of Arabidopsis A2-type cyclins are studied here and the authors show that one of the family members, CYCA2;3 plays a role in the stomatal cell lineage through direct control of its expression: the FOUR LIPS/MYB124 and MYB88 TFs repress CYCA2;3 expression. To my knowledge, this is the second report of a direct link between a developmental program and the expression of a cell cycle gene. In this sense, although not absolutely novel, this works constitutes a valuable addition that reinforces the concept of the direct control of cell division by developmental cues. While this is true, the manuscript, at least in its present form, lacks focus since various organs and cellular processes are studied, in some cases superficially. This leads to claim several conclusions that are insufficiently justified. In short, the part on the stomatal phenotype is strong but a similar depth should be reached in other sections. If this is not the case, some parts (root meristem, endoreduplication or vascular proliferation) are not well connected to each other or to the main axes of the manuscript.

Comments
1. What is the expression pattern of individual CYCA genes? A comprehensive study should be shown; otherwise the correlation between expression and phenotypes is difficult to demonstrate. A detailed study of the spatial and temporal expression pattern is necessary. For example, expression of CYCA2;3 seems to be restricted to the stomatal lineage. Does this mean that it is exclusively expressed in these cell types? Is it expressed in other cell types in the leaf? How its expression pattern correlates with the some phenotypes, e.g. endoreduplication? This comment also applies to data shown in Fig. 4A, which should be extended to other genes.

2. In some cases the multiple cyca mutants do not show a very consistent phenotype (see Fig. 1A). The triple cyca2;124 has almost no effect whereas the cyca2;234 shows a strong phenotype. Does this suggest that CYCA2;3 is the most relevant for the process under study? Is this the case for other processes (endoreduplication, stomatal development)?

3. Although the expression profile in the cell cycle would be consistent with a role of CYCA in G2/M, the lack of appropriate markers, earlier in the cell cycle, formally precludes the strong statements (page 7) in the manuscript. How do the authors rule out that CYCA play a role in S-phase?

4. The role in endoreduplication is not particularly novel, based on the published data involving CDKB.

5. Fig 4A. Labeling at late stages of stomatal development disappear. Could you explain this pattern?

6. It seems that a quadruple cyca2 mutant is used only for some experiments. Is there any reason for that? Why they are not used throughout the study for the various processes analyzed?

Referee #2 (Remarks to the Author):

The paper by Vanneste and Coppens et al. presents the characterization of a cyclin family in Arabidopsis. Due to large cyclin families in plants and an apparent high redundancy between cyclins in one family - as also demonstrated in this study - functional studies are challenging and therefore rare, yet they are key for understanding the plant cell cycle. Thus, this paper is important for a wider audience and adds an essential data set to the field.

The authors show that A2-type cyclins are vital regulators of G2-M transition in plants by observing that double or triple mutants are compromised in cell proliferation and concomitantly display and increase in endoreplication, a cell-cycle variant in which cells repeatedly go through S-phase but

 

skip mitosis.

A focal point of the paper is analysis of A2-type cyclins in the formation of stomata. The authors show that these cyclins redundantly control the last division of stomata precursor cell (guard mother cell) to divide symmetrically into the two guard cells that comprise a stoma.

Previously, it has been found that FLP and MYB88 control the expression of B1-type CDKs (Xie et al., Plant Cell 2010). In addition, B1-type cyclins have been found to bind to A2-type cyclins and a recent study by Boudolf et al. (Plant Phys 2010) has shown that B1-type CDKs and A2-type cyclins act together to promote cell division and repress endoreplication. Thus, this paper completes our understanding of the function of A2-type cyclins as a partern of B1-type CDKs and their concomitant repression by FLP and MYB88 at the end of stomata development.

Thus, conceptually this paper merits EMBO journal but I have a rather long list of minor comments that I feel need to be addressed to also reach the technical level required for a publication in EMBO.

1. The alleles used should be better described. Presumably the authors have done this and thus, this could be quickly addressed. In particular, it is important to know whether the mutants are null and the authors may show a supplementary file providing the molecularly confirmed insertion site and qRT expression data on the individual alleles used.
2. Do the authors have a specific system why they used certain triple combinations for some and not other aspects of their mutant description.
3. To get an impression about the general phenotypes, all double, triple and especially the quintuple mutant (3Xa2-b11-b12) generated here should be documented with pictures showing the typical growth performance of these plants in the greenhouse.
4. The ChIP data (Figure 6) should be quantified by qPCR, especially in cases where promoter fragment 1 and 3 give a signal in the IP in the mutant similar to what is seen in the wild type. In addition, may show a positive and a negative control.
5. In general, the paper lacks quantification followed by statistical analyses of the traits analyzed. This should be done at least for the following cases:
a. The root growth presented in figure 1.
b. Cell production in figure 2C.
c. Venation pattern in figure 3D
d. percentage of aberrant stomata in figure 4B
e. description of the additive phenotype of cyca2-b1 mutants in figure 5
6. On page 3 the authors say : « ... only a few direct connections between these processes have been identified at the molecular level (Sozzani et al, 2010; Xie et al, 2010)... » The authors might want to include the work by Brownfield et al. in PLoS Genetics in which the group of David Twell shows that DUO1 (interestingly another MYB factor) is required for the expression of CYCB1;1 and ectopic CYCB1;1 expression can even partially rescue the duo1 mutant phenotype.
7. page 9: The authors conclude at the bottom of the page by saying: "...Thus, tissue-specific CYCA2 expression is required to couple cell division to differentiation in dividing guard mother cells.... « However, a few lines above they correctly state: « ...Yet, the aberrant cells attained a guard cell identity and formed single guard cells instead of a pair of guard cells.... ». This means to me that differentiation of the stomata can actually be uncoupled from cell proliferation, similar to the conclusions drawn by Boudolf et al. Plant Cell 2004.
8. The authors should provide some explanations why they think that CYCA2 and CDKB1 act redundantly taking into account that their main argumentation is that both A2 and B1 from active complexes (the genetics presented here would rather argue the opposite).
9. The authors might also want to discuss why ectopic expression of CYCA2, a G2-M regulator as demonstrated here, in guard cells that are in wild type arrested in G1 phase causes or contributes to overproliferation found in flp mutants, i.e. how is S-phase entry activated and why do these cells continue to proliferate?
10. What is the evidence that FLP and MYB88 are regulators of additional developmental aspects besides the pure control of cell proliferation?
11. page 9 check spelling, it should read: „they also expressed..."

Referee #3 (Remarks to the Author):

 

CycA2;3 has been previously proposed to dose-dependently inhibit endocycle, in complex with the mitosis specific plant CDKB1;1 and capable to trigger cell division. Within this manuscript the authors analyse the overlapping roles of 4 A2-type cyclins in different developmental settings (primary root meristem, lateral root, leaf venation and stomata development). They first show that CYCA2;1-4 have an overlapping expression domain in the primary root and correspondingly they convincingly show by producing multiple mutant combinations that CYCA2 cyclins are collectively required for cell proliferation, specifically for mitosis in roots, lateral roots and leaves. They also demonstrate that these A2-type cyclins are collectively inhibit the extent of endoreduplication. Besides the overlapping expression domains, they also noticed expression domains where certain members of A2-types cyclins are specific, CYCA2;1 and CYCA2;4 for veins and CYCA2;2 and CYCA2;3 for developing stomata. Correspondingly, they show altered occurrences of serration tips which they link to veins-associated proliferation, and an aberrant stomata division, respectively. They have analysed the role of CYCA2;2 and 3 during stomata development, and found that these cyclins are required for the final division of stomata and they need to be repressed by FLP to halt proliferation. The work is professionally carried out and well presented. The most significant finding is that a developmental regulator of stomata linage, FLP act to repress CYCA2;3 to restrict cell cycling and attain the developmentally specified two celled structure of stomata.

Comments
1. The title is wrong "Developmental regulation of CYCA2s contributes to plant morphogenesis" CycA2 does not contribute to morphogenesis, the manuscript shows that it is required for cell division.
2. In the introduction they discuss the cell cycle regulation to textbook levels while almost completely omit to introduce what we already know about CYCA2-type cyclins in plants, yet their study is a systematic dissection of their function.
3. They say: Components of the G1-to-S transition control cell proliferation and differentiation events in shoots (Dewitte et al, 2003; Dewitte et al, 2007) and roots (Caro etal, 2007; Sozzani et al, 2010; Wildwater et al, 2005), highlighting the key role of thistransition in the cell's decision to exit cell cycle and activate differentiation. However, differentiation does not preclude an active G1-to-S pathway, as some differentiating cell types are known to go through multiple rounds of DNA duplication without mitosis. (endoreduplication) (Melaragno et al, 1993). An active G1-to-S pathway in these cells argues for the G2-to-M transition as an additional target for developmental regulation of proliferation" This paragraph is wrong. Endoreduplication has a very different G1/S transition that proliferating cells, e.g. CYCD3;1 strongly inhibit endoreduplication.
4. Since CYCA2;1 and CYCA2;4 has overlapping vein and CYCA2;2 and 3 stomata expression, I am not sure why they have not analysed these double mutants.
5. 4C DNA content of one-celled stomata could be interpreted as an arrest in G2 rather than endoreduplication. This would indicate that stomata is not competent to undergo endoreduplication in spite of having low level of CYCA2 level.
6. On the bottom of page 9 they say "Thus, tissue-specific CYCA2 expression is required to couple cell division to differentiation in dividing guard mother cells." However, CYCA2 expression does not couple cell division to differentiation, but it is required for the cell divisions in the stomata linage up to the last cell division.
7. On page 13 in the discussion the lengthy arguments on what might be the plant MIF/MPF is speculative and unnecessary.
8. On page 14 they refer to animal literature that the transcription of A-type cyclins is repressed during differentiation. However, they do not cite the work of Yoshizumi et al Plant Cell. 2006 Oct;18(10):2452-68 that increased level of poliploid 1 (ILP1) a transcriptional repressor of CYCA2, and therefore has analogous function to FLP in other cells.
9. In summary they say "CycA2s are part of the mechanism that coordinates the switch between proliferation and differentiation" As commented above, CYCA2 does not seem to affect differentiation, both in the mutant and in FLP with high CYCA2 levels all the tested stomata differentiation markers are unaffected.

1st Revision - authors' response 31 May 2011

Referee #1 (Remarks to the Author):

 

*Vanneste et al. deals in this manuscript with a question of major relevance, understanding the coordination of cell proliferation and differentiation in morphogenesis. The family of Arabidopsis A2-type cyclins are studied here and the authors show that one of the family members, CYCA2;3 plays a role in the stomatal cell lineage through direct control of its expression: the FOUR LIPS/MYB124 and MYB88 TFs repress CYCA2;3 expression. To my knowledge, this is the second report of a direct link between a developmental program and the expression of a cell cycle gene. In this sense, although not absolutely novel, this works constitutes a valuable addition that reinforces the concept of the direct control of cell division by developmental cues. While this is true, the manuscript, at least in its present form, lacks focus since various organs and cellular processes are studied, in some cases superficially. This leads to claim several conclusions that are insufficiently justified.*
*In short, the part on the stomatal phenotype is strong but a similar depth should be reached in other sections. If this is not the case, some parts (root meristem, endoreduplication or vascular proliferation) are not well connected to each other or to the main axes of the manuscript.*

REPLY: We thank the reviewer for appreciating our contribution towards understanding the coordination of cell proliferation and differentiation in plant development. With the description of different phenotypes, albeit in some cases superficially, we hope to give readers a glimpse of the importance of CYCA2s in different developmental contexts. We have made several changes in an attempt to meet the reviewers'criticisms.

*Comments*
*1. What is the expression pattern of individual CYCA genes?*
*A comprehensive study should be shown; otherwise the correlation between expression and phenotypes is difficult to demonstrate. A detailed study of the spatial and temporal expression pattern is necessary. For example, expression of CYCA2;3 seems to be restricted to the stomatal lineage. Does this mean that it is exclusively expressed in these cell types?*
*Is it expressed in other cell types in the leaf? How its expression pattern correlates with the some phenotypes, e.g. endoreduplication?*
*This comment also applies to data shown in Fig. 4A, which should be extended to other genes (CYCA2;2, ...).*

REPLY:
We have included expression patterns of all 4 CYCA2s for the different developmental aspects adressed; root apical meristems (Figure S3), lateral root formation (Figure S3), a short time series of developing first leaves (Figure S6), details of developing serration tips in first leaves (Figure S9), young epidermal cells containing different stages of stomatal development (Figure S10) and for CYCA2;2 and CYCA2;3 expression in 4DAG first leaves (Figure S8)to compare with Figure 3A.

Figure S3 shows that all CYCA2s are expressed in root apical meristems and lateral roots. Nevertheless, the observed phenotypes suggest that CYCA2;3 and CYCA2;4 are the main contributors to root meristem activity and lateral root formation

Figure S6 shows different stages of leaf development, demonstrating that CYCA2;2 and CYCA2;3 are dynamically regulated during leaf development; being quite ubiquitously expressed at early stages and more restricted and at lower levels at later stages, while CYCA2;1 and CYCA2;4 are quite specific for vascular tissues. One interesting aspect of CYCA2;2 and CYCA2;3 expression is that there seems to be a dynamic front where their expression patterns switch from ubiquitous to more restricted to stomatal lineage and vascular tissues. This dynamic front could correlate with the gradual tip-to-base gradient in cell division and switch to endoreduplication.

Figure S9 shows that CYCA2;2 and CYCA2;3 are broadly expressed in the margin of developing serration tips, while CYCA2;1 and CYCA2;4 are restricted to the vascular tissues in this area. This correlates well to the observation that additional mutation of cyca2;1 in cyca2;234 triple mutants does not enhance the serration tip phenotype.

Figure S10 shows that CYCA2;2 and CYCA2;3 are expressed in the stomatal lineage, whereas no expression of CYCA2;1 and CYCA2;4 could be detected in these cell types. Interestingly, cyca2;3 single mutants showed already some aberrant stomata, and this phenotype penetrance was dramatically increased by additional mutation of cyca2;2 fitting to their stomatal expression.

 

Figure S8 shows expression patterns of CYCA2;2 and CYCA2;3 in 4DAG first leaves. A developmental stage comparable to those depicted in Figure 3A. One can notice stomatal expression in the distal region of the leaf and more ubiquitous expression in subepidermal cells of the proximal region of the leaf. This also shows that these genes could contribute to vascular proliferation as their ubiquitous expression includes the vascular progenitor cells.

In summary, the expression patterns of different CYCA2s are in part overlapping and in part specific. This being said, "specific" should be regarded in a broad sense, meaning, a markedly high expression in a given developmental context. Also, their expression can change over time. This suggests complex developmental regulation and specificity. Although the expression patterns correlate quite well with the main contributing CYCA2s, it is still possible that other CYCA2s become ectopically expressed outside its normal expression domain to compensate for the loss of one or more others. Such a compensation mechanism was previously shown for auxin transport proteins of the PIN family (Vieten et al., 2005 Development), however, this type of functional cross-regulation was not addressed.

*2. In some cases the multiple cyca mutants do not show a very consistent phenotype (see Fig. 1A). The triple cyca2;124 has almost no effect whereas the cyca2;234 shows a strong phenotype. Does this suggest that CYCA2;3 is the most relevant for the process under study? Is this the case for other processes (endoreduplication, stomatal development)?*

REPLY: The reviewer has accurately observed that there are tremendous differences in phenotypic penetrance among the different triple mutants. In the case of root meristem size, lateral root formation, endoreduplication and stomatal development, CYCA2;3 seems to be the most relevant CYCA2 member. However, the key is in the combinations. We adressed additivity and synergism in greatest detail for the SGC phenotype. In this case, in single cyca2;3 mutant alleles ~2% of SGCs could be observed. Double mutants containing cyca2;3 mutation, increased this frequency dramatically (16-20%) and triple mutants containing cyca2;3 mutation had up to 94% of SGCs. Interestingly, not all synergistic interaction fit to the expression domains of CYCA2s in WT, which suggests that in some cases CYCA2s might be ectopically upregulated to compensate for the loss of another.

The analysis of the different triple mutants allows estimate the relative importance of the different CYCA2s to specific processes. Observed differences in penetrance can be explained in part by tissue-specific expression and relative expression levels. However, as we did not test for protein stability and differences in biochemical properties, we are unable to exclude additional effects on this level.

We included this in the discussion.

*3. Although the expression profile in the cell cycle would be consistent with a role of CYCA in G2/M, the lack of appropriate markers, earlier in the cell cycle, formally precludes the strong statements (page 7) in the manuscript. How do the authors rule out that CYCA play a role in S-phase?*

REPLY
We thank the reviewer for pointing out that we did not explain satisfactorily that our data does not allow to preclude a role for CYCA2s in S-phase. Indeed, the currently available data, and markers do not allow to rule out a role in S-phase. Given the analogy to the animal field, we believe that it is rather quite likely that CYCA2s would play a role in S-phase. Consistently with this, previously it has been shown that CYCA2-CDKA complexes can phosphorylate S-phase regulator, E2Fc, in vitro (del Pozo). We added this reference together with a statement that our analysis is not exclusive about roles in S-phase regulation.

*4. The role in endoreduplication is not particularly novel, based on the published data involving CDKB.*

REPLY:

 

Indeed, CYCA2;1, CYCA2;3 and CDKB1;1 have previously been implicated as negative regulators of endoreduplication (Imai et al 2006; Yoshizumi et al 2006; Boudolf et al 2009). However, the genetic redundancy among CYCA2s in endoreduplication was not demonstrated before, and therefore adds to our global understanding of endoreduplication in plants.

*5. Fig 4A. Labeling at late stages of stomatal development disappear. Could you explain this pattern?*

REPLY:
We removed the GUS patterns in Figure 4 because they were redundant with the detailed stomatal expression analysis in Figure 6. Overview pictures for all CYCA2s in the stomatal lineage are added in Figure S12. The expression patterns of CYCA2s at various steps in stomatal development are described in great detail in Figure 6. During guard mother cell division both CYCA2;3, FLP and MYB88 are highly expressed, and their expression disappears in mature guard cells. Given the ectopic expression of CYCA2;3 in flpmyb88 mutant background, we propose that FLP and MYB88 act to repress of CYCA2;3 expression after the guard mother cell has divided. We believe that through this repression, FLP/MYB88 transcription factors enforce a cell cycle exit, counteracting mitogenic stimuli. After full differentiation, mitogenic stimuli are no longer active and FLP/MYB88 expression disappears.

*6. It seems that a quadruple cyca2 mutant is used only for some experiments. Is there any reason for that? Why they are not used throughout the study for the various processes analyzed?*

REPLY:
The quadruple mutant has very strong, pleiotropic effects on plant growth and development, hampering the analysis and interpretation of the data (Cfr Greenhouse pictures). The quadruple mutant was particularly relevant in the analysis of vascular development, as it provided added value in the interpretation of specific expression of CYCA2;1 in vascular tissues.

Referee #2 (Remarks to the Author):

*The paper by Vanneste and Coppens et al. presents the characterization of a cyclin family in Arabidopsis. Due to large cyclin families in plants and an apparent high redundancy between cyclins in one family - as also demonstrated in this study - functional studies are challenging and therefore rare, yet they are key for understanding the plant cell cycle. Thus, this paper is important for a wider audience and adds an essential data set to the field.*

*The authors show that A2-type cyclins are vital regulators of G2-M transition in plants by observing that double or triple mutants are compromised in cell proliferation and concomitantly display an increase in endoreplication, a cell-cycle variant in which cells repeatedly go through S-phase but skip mitosis.*

*A focal point of the paper is analysis of A2-type cyclins in the formation of stomata. The authors show that these cyclins redundantly control the last division of stomata precursor cell (guard mother cell) to divide symmetrically into the two guard cells that comprise a stoma.*

*Previously, it has been found that FLP and MYB88 control the expression of B1-type CDKs (Xie et al., Plant Cell 2010). In addition, B1-type cyclins have been found to bind to A2-type cyclins and a recent study by Boudolf et al. (Plant Phys 2010) has shown that B1-type CDKs and A2-type cyclins act together to promote cell division and repress endoreplication. Thus, this paper completes our understanding of the function of A2-type cyclins as a partern of B1-type CDKs and their concomitant repression by FLP and MYB88 at the end of stomata development.*

*Thus, conceptually this paper merits EMBO journal but I have a rather long list of minor comments that I feel need to be addressed to also reach the technical level required for a publication in EMBO.*

REPLY: We thank the reviewer for a positive and constructive evaluation of our work

 

*1. The alleles used should be better described. Presumably the authors have done this and thus, this could be quickly addressed. In particular, it is important to know whether the mutants are null and the authors may show a supplementary file providing the molecularly confirmed insertion site and qRT expression data on the individual alleles used.*

REPLY:
We included in supplementary information gels showing examples of genotyping PCRs, for which the T-DNA flanking sequence was determined based on the amplicon derived from T-DNA specific primers and a gene-specific primer. The T-DNA derived amplicon was sequenced using the T-DNA specific primer, to determine the exact insertion site.
We also included RT-PCRs on cDNAs from each single mutant demonstrating that a T-DNA spanning transcript could not be formed, and thus no full-length transcript could be detected.
We further characterized the transcripts by Q-RT-PCR revealing that some of these mutants express truncated mRNAs, which could result in a partially functional protein, while others did not show any remnant expression. Other mutant alleles (flp, myb88, cdkb1;1) were previously characterised at the molecular level (Lai et al 2005; Xie et al 2010)

*2. Do the authors have a specific system why they used certain triple combinations for some and not other aspects of their mutant description.*

REPLY: We performed most experiments with triple mutant combinations cyca2;124 (which has very subtile phenotypes), cyca2;134 and cyca2;234, which show different degrees of phenotype penetrance. This allows to get a glimpse of the genetic complexity of the CYCA2 redundancy. Moreover, as the cyca2;1234 quadruple mutant is severely defective in growth, it is difficult to adress if the observed phenotypes are a result of a defect in development or rather an effect on cell proliferation or a combination of both. Therefore, we preferred the use of triple mutants growing at a similar rate to do phenotype analyses. For the vascular proliferation, the cyca2;1234 quadruple mutant provided added value.

*3. To get an impression about the general phenotypes, all double, triple and especially the quintuple mutant (3Xa2-b11-b12) generated here should be documented with pictures showing the typical growth performance of these*
*plants in the greenhouse.*

REPLY: We included pictures of representative single, double, triple and quadruple mutants in the green house(Figures S2). We regret to have noticed a mislabeling; the data refering to a quintuple mutant (3xa2-b11-b12) should in fact have been the quadruple mutant 3xa2-b11. We severely regret this event. Fortunately, it does not affect any of our conclusions. We included greenhouse performance pictures comparing WT, cdkb1;1, cyca2;234 and cyca2;234/cdkb1;1 (Figure S11), each of them after having re-checked their genotypes.

*4. The ChIP data (Figure 6) should be quantified by qPCR, especially in cases where promoter fragment 1 and 3 give a signal in the IP in the mutant similar to what is seen in the wild type. In addition, may show a positive and a negative control.*

REPLY:
We replaced the gel pictures in Figure 6 by qPCR data with 3 probes representing 3 regions of the CYCA2;3 promoter. The data demonstrate unequivocally that FLP/MYB88 can bind to chromatin of the CYCA2;3 promoter, with highest affinity to fragment P2.

*5. In general, the paper lacks quantification followed by statistical analyses of the traits analyzed. This should be done at least for the following cases:*

*a. The root growth presented in figure 1.*

REPLY: In Figure 1 we show representative images of root meristem size as indicated by fewer cells that are elongated beyond isodiametrical size.
We included as supplemental data alternative quantifications plus suitable statistical significances of meristem size in the mutants: 1) the number of cells between quiescence centre and first elongating

 

cell in meristem (Figure S5A) the corresponding distance (QC to first elongating cortex cell) (Figure S5B). The defect in KNOLLE expression supports this notion. We added as supplemental data a graph quantifying the number of cell plates labeled with KNOLLE signal, demonstrating that fewer cells are undergoing cytokinesis in the meristem of cyca2 triple mutants (Figure S5C).

*b. Cell production in figure 2C.*

REPLY: Our current setup doesn't allow statistics on cell division rates because we used destructive harvesting of a different set of replicate leaves each day. We then used all replica's to make a single polynomial fit, which is then used to calculate the derivative that gives the cell division rates. The only way to achieve this would be to repeat the experiment several times and average across multiple experiments. Since each experiment takes about a full month of laborious drawing, this effort is not proportional to the additional information obtained.
However, to allow for the readers to somehow evaluate the variability in the data, we included in supplemental data the evolution of cell production in the leaves, including error bars (Figure S7).

*c. Venation pattern in figure 3D*

REPLY: We included error bars in the figure. Statistics and significances are described in the figure legend in greater detail.

*d. percentage of aberrant stomata in figure 4B*

REPLY: We performed a Fisher's exact test to test for statistical significance.

*e. description of the additive phenotype of cyca2-b1 mutants in figure 5*

REPLY: We included a quantification of the additive phenotype of cyca2;234/cdbk1;1 to compare to cyca2;234 and cdkb1;1 and included a Fisher's exact test to test the significance of cdkb1;1 on SGC frequency in cyca2;234.

*6. On page 3 the authors say : « ... only a few direct connections between these processes have been identified at the molecular level (Sozzani et al, 2010; Xie et al, 2010)... » The authors might want to include the work by Brownfield et al. in PLoS Genetics in which the group of David Twell shows that DUO1 (interestingly another MYB factor) is required for the expression of CYCB1;1 and ectopic CYCB1;1 expression can even partially rescue the duo1 mutant phenotype.*

REPLY: We thank the reviewer for providing an additional interesting reference. We included this reference in the introduction. Yet, this paper does not show experimental evidence of direct interaction senso strictu, but shows developmental control (direct or indirect) of CYCB1;1 by DUO1 transcription factors. Therefore, we removed "direct" to allow us to use this reference in the text

*7. page 9: The authors conclude at the bottom of the page by saying: "...Thus, tissue-specific CYCA2 expression is required to couple cell division to differentiation in dividing guard mother cells.... « However, a few lines above they correctly state: « ...Yet, the aberrant cells attained a guard cell identity and formed single guard cells instead of a pair of guard cells.... ». This means to me that differentiation of the stomata can actually be uncoupled from cell proliferation, similar to the conclusions drawn by Boudolf et al. Plant Cell 2004.*

REPLY: We completely agree with the reviewer, however, it seems that our wording was somewhat ambiguous, therefore we changed the conclusion of this paragraph: "Collectively these data demonstrate that CYCA2s are synergistically required for the symmetric division that is a prerequisite for stomatal formation, and that acquisition of guard cell identity occurs independently from guard mother cell division."

*8. The authors should provide some explanations why they think that CYCA2 and CDKB1 act redundantly taking into account that their main argumentation is that both A2 and B1 from active complexes (the genetics presented here would rather argue the opposite).*

 

REPLY: Indeed, the term redundancy was not used correctly in this complex genetic background. Since mutants in either CYCA2s and CDKB1s cause single guard cell phenotype, and a higher penetrance in this phenotype in the cyca2;234 x cdkb1;1 compared to either cyca2;234 and cdkb1;1 suggest they contribute to the same pathway. As they are both components of CDK complexes, and their ability to interact, suggests that they thus contribute to the CDK activity of which a certain treshold should be met to complete the guard mother cell division. The fact that we cannot generate plants lacking normal stomatal complexes with either cyca2 or cdkb1 mutant combinations suggests that other cyclins and cdks can take over the function of the mutated cyclins or cdks. We changed the title of this paragraph to "CYCA2s and CDKB1;1 contribute synergistically to the CDK activity required for guard mother cell division" for more accuracy

*9. The authors might also want to discuss why ectopic expression of CYCA2, a G2-M regulator as demonstrated here, in guard cells that are in wild type arrested in G1 phase causes or contributes to overproliferation found in flp mutants, i.e. how is S-phase entry activated and why do these cells continue to proliferate?*

REPLY: As demonstrated by Xie et al 2010, FLP/MYB88 transcription factors target many more genes besides CYCA2;3. Among them several cell cycle regulators, including G1/S-phase related genes, such as CYCD4;1, CDC6a, CDC6b, but also other CYCB1,3 and CDKA;1... Therefore we believe that deregulation of CYCA2s is required but not sufficient to explain the flp phenotype.

*10. What is the evidence that FLP and MYB88 are regulators of additional developmental aspects besides the pure control of cell proliferation?*

Indeed, FLP and MYB88 are best known for their role in restricting proliferation in the stomatal lineage. However, more than 500 genes were found to be misregulated in flpmyb88 mutants; among them many genes related to stress conditions. This observation was supported with an inability to respond to drought stress and an impaired ABA response (Xie et al 2010; Plant J). Together, these data highlight the importance of FLP and MYB88 in a broader perspective than regulation of stomatal proliferation. However, so far, additional developmental roles for FLP and MYB88 remain unexplored.

*11. page 9 check spelling, it should read: „they also expressed..."*

REPLY: We corrected this mistake

Referee #3 (Remarks to the Author):

*CycA2;3 has been previously proposed to dose-dependently inhibit endocycle, in complex with the mitosis specific plant CDKB1;1 and capable to trigger cell division. Within this manuscript the authors analyse the overlapping roles of 4 A2-type cyclins in different developmental settings (primary root meristem, lateral root, leaf venation and stomata development). They first show that CYCA2;1-4 have an overlapping expression domain in the primary root and correspondingly they convincingly show by producing multiple mutant combinations that CYCA2 cyclins are collectively required for cell proliferation, specifically for mitosis in roots, lateral roots and leaves. They also demonstrate that these A2-type cyclins are collectively inhibit the extent of endoreduplication. Besides the overlapping expression domains, they also noticed expression domains where certain members of A2-types cyclins are specific, CYCA2;1 and CYCA2;4 for veins and CYCA2;2 and CYCA2;3 for developing stomata.*
*Correspondingly, they show altered occurrences of serration tips which they link to veins-associated proliferation, and an aberrant stomata division, respectively. They have analysed the role of CYCA2;2 and 3 during stomata development, and found that these cyclins are required for the final division of stomata and they need to be repressed by FLP to halt proliferation. The work is professionally carried out and well presented. The most significant finding is that a developmental regulator of stomata linage, FLP act to repress CYCA2;3 to restrict cell cycling and attain the developmentally specified two celled structure of stomata.*

REPLY: We thank the reviewer for a positive evaluation of our work.

 

*Comments*
*1. The title is wrong "Developmental regulation of CYCA2s contributes to plant morphogenesis" CycA2 does not contribute to morphogenesis, the manuscript shows that it is required for cell division.*

REPLY: We agree that CYCA2s are required for cell division. Given that morphogenesis relies on a spatio-temporal control of cell division and that CYCA2s are targets of developmental signals, we feel it was fair to say that the developmental regulation of CYCA2s contributes to tissue-specific proliferation and thus, by extension, to morphogenesis. We changed the title to meet the reviewer's criticism

*2. In the introduction they discuss the cell cycle regulation to textbook levels while almost completely omit to introduce what we already know about CYCA2-type cyclins in plants, yet their study is a systematic dissection of their function.*

REPLY: We agree that we did not comprehensively summarise the state-of-the-art of what we know about CYCA2-type cyclins in plants. Therefore, we included a new paragraph summarizing the latest most important findings on plant CYCA2s.

3. They say: Components of the G1-to-S transition control cell proliferation and differentiation events in shoots (Dewitte et al, 2003; Dewitte et al, 2007) and roots (Caro etal, 2007; Sozzani et al, 2010; Wildwater et al, 2005), highlighting the key role of thistransition in the cell's decision to exit cell cycle and activate differentiation. However, differentiation does not preclude an active G1-to-S pathway, as some differentiating cell types are known to go through multiple rounds of DNA duplication without mitosis. (endoreduplication) (Melaragno et al, 1993). An active G1-to-S pathway in these cells argues for the G2-to-M transition as an additional target for developmental regulation of proliferation" This paragraph is wrong. Endoreduplication has a very different G1/S transition that proliferating cells, e.g. CYCD3;1 strongly inhibit endoreduplication.

REPLY: We understand the concern raised by the reviewer. Indeed, endoreduplication cycles could involve specific S-phase components. Yet, in this paragraph we did not want to claim that S phase regulation is the same in proliferating and endoreduplicating cells, but rather that the absence of mitosis correlates with endoreduplication. Therefore, downregulation of G2-to-M regulation might be part of a mechanism coordinating the switch between proliferation and endoreduplication. We changed this paragraph accordingly.

*4. Since CYCA2;1 and CYCA2;4 has overlapping vein and CYCA2;2 and 3 stomata expression, I am not sure why they have not analysed these double mutants.*

REPLY:
While it is true that CYCA2;1 and A2;4 have overlapping expression in the veins, this feature is by no means limited to A2;1 and A2;4. In Figure S8 we shows expression patterns of CYCA2;2 and CYCA2;3 in 4DAG first leaves. A developmental stage comparable to those depicted in Figure 3A. One can notice stomatal expression in the distal region of the leaf and more ubiquitous expression in subepidermal cells of the proximal region of the leaf. This suggests that these genes could contribute to vascular proliferation as their ubiquitous expression includes the vascular progenitor cells Figures 6 and S12 show that CYCA2;2 and CYCA2;3 are expressed in the stomatal lineage, whereas no expression of CYCA2;1 and CYCA2;4 could be detected in these cell types. In Table SI we provide a detailed analysis of multiple single, double and triple mutant combinations addressing genetic redundancy among CYCA2s in stomatal cell division. Interestingly, cyca2;3 single mutants showed already some aberrant stomata, and this phenotype penetrance was dramatically increased by additional mutation of cyca2;2 fitting to their stomatal expression. Although we could never find CYCA2;4 expression in the stomatal lineage, cyca2;34 mutants had a higher frequency than cyca2;3 alone, suggesting that CYCA2;4 might become ectopically expressed when cyca2;3 is mutated.

*5. 4C DNA content of one-celled stomata could be interpreted as an arrest in G2 rather than endoreduplication. This would indicate that stomata is not competent to undergo endoreduplication in spite of having low level of CYCA2 level.*

 

REPLY: We agree with the reviewer, and to avoid the potential mis- interpretation of endoreduplication we changed the text accordingly. Moreover, for endoreduplication, one would require additional signals that trigger re-entry into S-phase

*6. On the bottom of page 9 they say "Thus, tissue-specific CYCA2 expression is required to couple cell division to differentiation in dividing guard mother cells." However, CYCA2 expression does not couple cell division to differentiation, but it is required for the cell divisions in the stomata linage up to the last cell division.*

REPLY:
We completely agree with the reviewer, however, it seems that our wording was somewhat ambiguous, therefore we reworded the conclusion of this paragraph: “Collectively these data demonstrate that CYCA2s are synergistically required for the symmetric division that is a prerequisite for stomatal formation, and that acquisition of guard cell identity occurs independently from guard mother cell division.”

*7. On page 13 in the discussion the lengthy arguments on what might be the plant MIF/MPF is speculative and unnecessary.*

We shortened this paragraph dramatically

*8. On page 14 they refer to animal literature that the transcription of A-type cyclins is repressed during differentiation. However, they do not cite the work of Yoshizumi et al Plant Cell. 2006 Oct;18(10):2452-68 that increased level of poliploid 1 (ILP1) a transcriptional repressor of CYCA2, and therefore has analogous function to FLP in other cells.*

REPLY: We included this reference by adding “Previously, INCREASED LEVEL OF POLYPLOIDY1 (ILP1) was found to act as repressor of CYCA2 expression (Yoshizumi et al 2006).” to the discussion.

*9. In summary they say "CycA2s are part of the mechanism that coordinates the switch between proliferation and differentiation" As commented above, CYCA2 does not seem to affect differentiation, both in the mutant and in FLP with high CYCA2 levels all the tested stomata differentiation markers are unaffected.*

REPLY: This paragraph was replaced in the new version of the discussion.

2nd Editorial Decision 22 June 2011

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referees #1 and 2 to review the revised manuscript and have now heard back from both of them. As you can see below, both referees support publication here. Referee #1 has a few minor suggested text changes. Take a look at them and see if they are correct. You can send us a modified word file by email and we will upload it for you. Once we resolve these last issues we will accept the paper for publication here.

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

This is a resubmission of the original manuscript submitted byVanneste et al. Here the authors have addressed satisfactorily my concerns, at least in most and more controversial points. The manuscript now reads much more fluently and the conclusions have been modified to those which can be strictly deduced from the results presented. I consider it a valuable addition to the plant field, with

implications on cell proliferation control in multicellular organisms, in general.

1. in page 11, authors please check whether the sentence referring to "... even less SGCs..." is correct or it should be "... even more SGCs".

2. Check the calls to supplementary figures describing the expression pattern of various CYCA2 genes.

Referee #2:

The authors have sufficiently addressed all of mine as well as the by other reviewers previously raised concerns. With this the manuscript presents a very solid and sound analysis of how the plant cell cycle is controlled by developmental regulators.

 