Transcriptional repression by MYB3R proteins regulates plant organ growth

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Editors: Alexander Kohlmaier and Andrea Leibfried

1st Editorial Decision 24 July 2014

Thank you for submitting your study "Transcriptional repression by MYB3R proteins is required for spatial and temporal regulation of cell division" for consideration in the EMBO Journal. First of all I have to apologize for not contacting you earlier with a decision. We have now received the reports of two expert reviewers, which you will find copied below. The third reviewer has not submitted their report, and despite multiple phone calls to this reviewer and repeated promises from the reviewer's side it has still not arrived. The editorial team decided to proceed to a decision in the interest of your time and chose to base the decision on the two reports already at hand. We can offer to forward the missing third report to you for your information, should it arrive at some point.

As you will see, the referees' specific comments are well-considered and constructive. We have to come to the conclusion, however, that your manuscript did not receive the necessary strong support by the referees to make it a candidate for publication in the EMBO Journal at this point.
While the referees acknowledge that your core findings are of interest in principle, they voice a number of substantial concerns and considerations. In particular, they were concerned that the presented evidence was not sufficiently comprehensive at this point to be able to conclusively argue that MYB3R-3/5/1 do function in a complex resembling the animal DREAM complex and that they indeed repressed G2/M genes in this function. The first concern was shared by both referees, and biochemical experiments were suggested to decisively determine association with other known DREAM complex members. The second concern seems rooted, in part, in the problem that no direct measure of the cell cycle phase was reported, and that the cellular origin of the mutant phenotypes was not sufficiently systematically and quantitatively determined, as referee #1 most clearly explains. For example, whether G2/M genes were de-repressed (or whether the mitotic cell division phase was just prolonged, or cells were arrested in G2/M) and how altered cell cycle activity was related to organ growth alterations remained unclear in the absence of more dedicated quantitative assays and systematic experiments. Moreover, the cell proliferation history was not tracked in the relevant developmental stages and during differentiation onset in the mutants. This and additional discrepancies in the different mutant combinations and epistasis analyses led to the concern that the function of the repressive Mybs in early leaf development might not be related to the proposed DREAM-like complex function. Finally, functional evidence was also requested to extend the existing ChIP data - addressing whether the repressive Mybs control E2F targets (and this possibly not only in quiescent cells, but also in cycling cells, which would be expected of DREAM complex function) might substantiate the interpretations made here.

Together, I am sorry not to be able to communicate more encouraging news at this point. I hope that you will find our referees' comments and suggestions helpful.

REFEREE COMMENTS

Referee #2:

Using combinations of loss-of-function mutations of plant MYB3R transcription factors the authors show that they are required for the regulation of certain G2/M-specific genes. They find MYB3R3 associated with RBR throughout leaf development and with E2F, E2FC and CDKA in older leaves. Based on these protein associations the authors speculate that MYB3R3 might be a subunit of a plant regulatory complex similar to the DREAM/dREAM complex in animals and Drosophila. These are interesting observations, but overall the work is fragmented and inconclusive and the manuscript is quite disorganized. At present the manuscript does not meet the quality and impact required for publication in EMBO Journal.

In particular, the authors must provide a systematic and quantitative description of the mutant phenotypes. Otherwise it is impossible to determine if equivalent material was used for the expression analyses and how the length of cell division and cell growth periods contribute to the different results. Based on the current data it is unclear how the rep-MYBs affect cell cycle/proliferation, cell expansion, and organ growth. The effects of the altered cell cycle for organ growth cannot be understood in an intuitive way, for example, in many cases altering the cell cycle results in smaller leaf or altered cell size. In particular, the results suggest that the rep-MYBs have different functions in early and late stages of leaf development and the function in early leaf development is clearly not related to a DREAM-like complex proposed by the authors. To unravel such functions requires precise and comprehensive analysis of the rep-MYB mutants. Ferjani et al. (2007, Plant Physiology) reported a comprehensive analysis on cell proliferation rate, size and expansion rate. Such precise quantification is needed to reveal the roles of rep-MYBs in early leaf development.

For example, based on the results in Fig. 4a the authors conclude that a root growth defect in ple-2 is complemented partially by the myb3r(1)/3/5 mutation and this is taken as proof for a genetic interaction. However, Fig. 7g shows that the myb3r(1)/3/5 mutation alone enhances root growth also in absence of ple-2. Moreover, the explanation for the partial complementation by increased expression of mutant PLE (Fig. S8b) is neither evident from the data nor mechanistically likely. Similarly, for the gene expression experiments in Fig. 2b the authors show images of wild type but not mutant plants. Some indications of mutant phenotypes are provided in Fig. 7d, but not for 9 and 15 day-old plants that were used for most of the experiments.
Based on the expression of selected genes "early" and "later" in development (Fig. 2b,c,d) the authors suggest that G2/M genes are de-repressed in differentiated cells. They do not exclude the possibility that such differences could also be a consequence of prolonged cell division activity and/or prolonged G2/M phases during the cell cycle. Most of the data shown in Figures 3 and S11 would also be consistent with this possibility.

Fig. 2a shows defects in cytokinesis in myb mutants, but again without explanation or quantitative information. It appears that the guard cell phenotype depends on simultaneous loss of MYB3R1/4 and is not affected by loss of MYB3R3/5 function. However, loss of MYB3R3/5 function increases KN expression, which according to a previous publication from co-authors should complement the defect. The authors acknowledge that these "data are more complicated", but they make no attempt to reconcile this discrepancy and such inconclusive results do not strengthen their conclusions. A quantitative evaluation of the different types of cytokinesis defects (e.g. as shown in Fig. 4b) together with tracking the cell division history of cells throughout relevant developmental stages in the different mutants could substantiate the gene expression data to provide a better understanding of the underlying molecular processes. For example, the reporter lines could be used for a systematic analysis of differences in leaf development (e.g. by observing the CYCB1;2:YFP line at different leaf ages in wild type and mutants). The MYC3R3:GFP line will provide useful information on MYC expression patterns (Fig. S10 suggests that this MYC is not expressed in mature cells; in this context a MYB3R3:GFP and other MYB promoter constructs would be helpful for evaluation of MYB expression).

The gene expression data need to be shown and discussed in more detail. The authors apparently have data from a number of array experiments, but in Materials and Methods only one is described in some detail for 15 day-old myb3r1/3/5 plants. Fig. 1c shows data for four mutants (including myb3r1/3/5) at day 9. Fig. S3 contains additional four mutants at day 9. Fig. 2c contains an experiment with myb3r1/3/5 plants after 5 days. No detailed meta-information is provided for either of these experiments. The authors only show a list of the 30 most up-regulated genes in Table S1 and two heat maps displaying an array of G2/M-specific genes without any further annotation. This information is insufficient and does not support their claims. It is necessary to provide the total number and lists of deregulated genes to evaluate if they are truly G2M phase-enriched genes, if other cell cycle phases may be affected, or if potential regulatory genes are deregulated. The presented heat maps show a complicated pattern of up- and down-regulated genes even among the G2/M genes, suggesting that all MYB factors have both positive and negative effects on some genes. In a previous publication the authors reported high variability among replicates of myb3r1/5 plants. Without the corresponding information for the present datasets it is impossible to determine if the high variability indicates different adaptations to the loss of the MYB proteins and thus help to distinguish between direct effects from indirect effects on gene expression.

The direct association of MYB3R3 with selected promoters of up-regulated genes is convincing, although a ChIP with Arabidopsis plants expressing GFP as a negative control is missing for the data in Fig. 5. However, the ChIP data alone are not evidence that binding contributes to regulation. The MSA motif occurs very frequently in promoters and likely also in genes that show no or an opposite response to MYB loss of function. Examples of such genes must be included in the analysis.

Fig. 6 shows that MYB3R3 can be co-precipitated with RBR throughout the leaf development, but appears to be associated with CDKA and E2FC only later in leaf development. This alone is not evidence for a plant DREAM-like complex involved in the repression of certain G2/M-specific genes. If a DREAM complex is proposed, the size of MYB-containing complexes should be investigated using gel filtration together with a panel of antibodies. The DREAM/dREAM complex has nine core subunits, DP, RB-like pocket protein, RB binding protein, E2F, MYB, LIN9, LIN54, LN37, LIN52. It is currently unknown if LIN9, LIN54, LN37 and LIN52 have homologs in plants but at least DP and MSI1 could be checked. The authors show that CDKA can also be co-precipitated with MYB3R3, but CDK is not a DREAM complex subunit in animals. It is also not evident from the data if MYB3R3, E2FC, RBR and CDKA all function in a single complex to repress the expression of certain G2/M genes in quiescent cells. Furthermore, in animals many E2F targeted cell cycle genes are regulated via the DREAM complex, thus it is reasonable to test not only MYB-target genes, but E2F-target genes as well. The discussion of DREAM is focused mainly...
on similarities to the mammalian complex and regulation of developmental genes in quiescent cells. But as pointed out by one of the coauthors in a recent paper, the Drosophila dREAM complex is also acting in dividing cells.

The first half of the Introduction discusses the roles of cell proliferation on leaf development, the importance of DNA endoreduplication, and scheduled cease of cell proliferation. However, the data do not show a convincing effect of rep-MYB loss-of-function on the timing of cell proliferation in leaves. Thus, the Introduction is misleading and should be rewritten.

Minor point:

The authors previously reported that MYB3R1 functions as an activator because loss of MYB3R1/4 function resulted in the down-regulation of certain G2/M specific genes. Since the myb3r1/3/5 triple mutant enhanced the up-regulation of certain G2/M specific genes, the authors now argue that MYB3R1 could be redundant with MYB3R3/5 and might function as repressor. However, it is unlikely that all de-regulated genes are direct targets of MYB3R1, thus it seems premature to define MYB3R1 as a repressor or activator based on the number of de-regulated genes. If authors want to define MYB3R1 as a repressor or an activator, they should identify several of direct targets of MYB3R1 and investigate their expression pattern in the loss-of-function mutant. MYBs have highly conserved structures and might cooperate with other proteins to control gene expressions. Thus, imbalanced populations/levels of MYBs might cause changes in binding preferences or efficiencies.

Referee #3:

This manuscript describes the contribution of repressor MYB transcription factors to repress G2/M genes. A variety of assays are used to support the redundant role of MYB3R3, MYB3R3 and MYB3R1 in suppressing G2/M gene expression. Assays include microarray, YFP-fusions of several G2/M genes including CYCB1, EDE1, and NACK1 as well as assessment for defects in cytokinesis (Fig 2A), and increased G2/M gene expression in 15-day-old leaves.

The evidence for increased frequency for endo-duplication is modest with reported large nuclei in cells expressing CYCB1;2-YFP (Fig 3A). A direct stain for DNA content may be a better indicator of increased ploidy.

On page 9 it was indicated: "Compared to wild type, we observed a larger proportion of cells expressing the CYCB1;2-YFP marker in root meristems of the myb3r1/3/5 mutant (Fig 3B). This suggests that loss of Rep-MYBs resulted in elevated gene expression in cells outside the G2/M phase and normal expression window of the CYCB1;2-YFP marker."

No direct measure of the cell cycle phase was reported. Although it is a reasonable model to assume that loss of the repressor MYB results in expression of G2/M genes during other phases of the cell cycle, it would be very helpful to demonstrate this. It is possible that cells become arrested in G2/M and this accounts for the increased frequency. Perhaps co-expression of a G1/S gene with a G2/M gene could be evaluated.

Figure 6. This figure reports co-immunoprecipitation of GFP-MYB3R3 with RBR, CDKA:1 and E2FC. It is not clear why the interaction is variable at the different leaf ages. An important control, the immunoprecipitation of the target GFP-MYB3R3 is missing. This is an important control since the ChIP for GFP-MYB3R3 uses the same antibody. Additional controls to demonstrate the specificity of the interaction between GFP-MYB3R3 and RBR and E2FC that would be useful include the activator E2Fs, E2FA and E2FB. Another option to demonstrate specificity for the interaction is to test if MYB3R5, MYB3R1, or MYB3R4 can bind to RBR, E2FC and CDKA. Alternatively, genetic evidence for interaction between MYB3R3 and RBR or E2FC would be helpful.

While much of the abstract, introduction and discussion is focused on the similarity between the findings in this manuscript and the mammalian DREAM and drosophila dREAM complex, it would be very helpful to test if GFP-MYB3R3 binds to a homologue of the DREAM/dREAM complex such as LIN9, i.e. ALWAYS EARLY or other candidate homologues.
Thank you very much for handling our manuscript EMBOJ-2014-89186 ("Transcriptional repression by R1R2R3-Myb proteins is required for spatial and temporal regulation of cell division in Arabidopsis"). I have carefully thought through and discussed with co-authors the comments you and the two experts made, and we have come to the conclusion that most criticisms can be addressed by pointing to the data already submitted in the manuscript, or data that we have collected meanwhile; or they can be addressed relatively easily by additional experiments. You do not explicitly state the decision on the manuscript, but we would like to get an opportunity to revise and resubmit the work. Below we provide a short response but we are happy to make a detailed point-by-point response for you and the referees.

1) One major area of criticism relates to whether the myb1/3/5 triple mutant (loss of all repressor MYBs) leads to extended G2/M phase in the cell cycle and/or prolongation of cell division during development. We agree that these possibilities should be resolved to conclusively show that ectopic expression of G2/M genes occurs in cells outside of G2/M both during the cell cycle in proliferating cells as well as in post-mitotic cells during development, when all the repressor MYBs are mutated. We showed in Fig. 7E, by flow cytometry, that the ratio of 2C and 4C cells is not significantly affected by myb1/3/5 mutation in young leaves (8d), when cell proliferation is still ongoing; if anything it is slightly less than that in wild type (this might not be evident in the condensed presentation of data in Fig. 7E, but the flow cytometric data can be presented as a histogram). The unaltered G2 phase measured by flow cytometry indicates that there is no prolonged G2/M. Moreover, we have performed kinematic analysis during leaf development, which shows cell proliferation rates are similar between wild type and myb1/3/5 mutant. These data exclude the possibility of prolonged cell division during development, as requested by reviewer #2, through systematic analysis of cell proliferation history during development. The kinematic data can be added into the revised manuscript. These data together with other data already presented, such as comparing the expression of genes that are targets (EDE1, CYCB1;2) and not targets of MYBs (CYCD3;1), see Fig. 2B, in situ observation of CYCB1;2-GFP during development that changes from a cell cycle dependent patchy pattern to a more even expression all point to a de-repressed gene expression.

2) The second area that came up in the comments of both referees is whether we have provided enough evidence for the existence of the DREAM complex in plants. As referee #2 rightly points out, there are only certain components of DREAM that are conserved from animals to plants, and there is clear functional specialization as well in different organisms, e.g. Drosophila and worm. We show association of three important regulators by co-IP, MYB, E2F and RBR. These are the components for which we have tools to study them (available antibodies and/or tagged lines). We did not present the data, but we have evidence for specificities in developmental regulation of repressor and activator MYB association with RBR, during differentiation vs cell proliferation, and we also see differences in association with E2FC and E2FB. As asked by reviewer #3, these data can be added to the revised manuscript. Recently we started to perform mass spectrometry-based identification of DREAM complex components through GFP pull downs of RBR, E2Fs and MYBs, and we detected some other conserved DREAM complex components in these experiments such as the presence of LIN9 homologs (ALY genes) and some HDACs.

3) The third area was the analysis of MYB target genes. We have repeated the microarray analysis for the myb1/3/5 triple mutant and we focused on the mitotic genes that we have previously shown to be expressed specifically during G2/M. However, we can certainly present a more complete analysis of these microarrays including the list of differentially expressed genes, unbiased GO overrepresentation analysis. One main area of interest is whether MYBs and E2Fs have common targets. We have performed ChIP-seq experiments and obtained strong evidence that MYB3R3 binds to both G2/M genes and E2F target genes. These results further support the presence of a DREAM-like complex in plants, which contains both Myb and E2F.
We would very much appreciate your opinion on these comments. We strongly feel, that showing the first time in plants (1) through thorough genetic analysis of MYB repressor functions in development, (2) indications of complexes whereby RBR and E2Fs associate with MYBs and (3) have common target genes - would provide a valuable contribution to the EMBO J.

Thank you very much for your message. I appreciate a lot your detailed response to the referees' concerns and criticism. I agree that the additional data and experiments that you offer in your letter are substantive. I will be able to inform you about the editorial team's decision on your request within the next few days.

Alexander is on his summer leave and I am stepping in in the meantime. We have discussed a resubmission of your amended manuscript for potential re-review within the editorial team and we have agreed to send the revised version out for peer-review. Please submit your manuscript as a new submission to our journal via the online system. Please also provide a point-by-point response to the previously raised criticisms, as we will likely approach the same referees again.

Point-by-point response

We thank for the three reviewers and the editor for their input, suggestions and constructive criticism of the manuscript. We considered each of their points and revised the manuscript accordingly. Below we provide a point-by-point response for each comment.

1) Comments: It is not clear if GFP expression occurs outside of G2/M or just G2/M is prolonged in Rep-MYB mutants (Fig. 3 F-H).
(Comments from editor and both reviewers#2 and #3)
Response:
We did present data of DNA content analysis by flow cytometry of leaves at various stages of development in Fig. 7E. In the youngest leaves with proliferating cells (8 DAG), there are only 2C and 4C cells. The proportion of 4C cells (G2 DNA content) is not increased but rather it is less in myb3r1/3/5 mutant in comparison with wild type. The same data of DNA content analysis of leaves is now shown in Supplementary Fig. S21 plotted as the proportion of cells in each ploidy level that makes it easier to visualize the G1 and G2 cell cycle parameters. We also conducted additional flow cytometric cell-cycle phase measurement of root tips, which are comprised of only proliferating cells of 2C and 4C DNA content. The results are in full agreement with what we found in leaves and show that the proportion of 4C cells is significantly lower in myb3r1/3/5 than in wild type (please see new Supplementary Fig. S19E). We believe that these results reasonably exclude the possibilities of prolonged G2 phase or cell cycle arrest at G2 in the Rep-MYB mutant. Instead, the length of G2/M phase in myb3r1/3/5 is likely to be equivalent to or shorter than that in wild type. Therefore, we conclude that the increased proportion of cells expressing CYCB1;2-YFP is most likely caused by derepression of G2/M-specific genes in cells outside the G2/M in meristems and developing young organs rather than cell cycle arrest in G2.

2) Comments: Cell proliferation history should be examined quantitatively and systematically to show cellular basis of organ growth alterations in the Rep-MYB mutant.
(Comments from editor and both reviewers#2 and #3)
Response:
We performed additional kinematic leaf growth experiments to analyze time-course changes of leaf size, average cell size, cell number per leaves and calculated cell proliferation rate during leaf development (please see new Supplementary Fig. S20A). These data clearly show that there is only an increase in cell production at the earliest stages of leaf development shortly after initiation and this increased cell number is maintained without a further change in cell proliferation rate or prolonged cell proliferation in myb3r1/3/5 leaves, which reasonably excludes the possibility that prolonged expression of G2/M-specific genes (as shown in Fig. 2B, C, D) is simply due to prolonged duration of cell proliferation. Instead, it is more likely to be due to derepression of the G2/M-specific genes in post-mitotic cells. These cells being differentiated is further indicated by the lobbed pavement cell morphology and large nuclei indicative for high ploidy DNA content (Fig. 3A and G, Supplementary Fig. S12 and S13).

The data of kinematic analysis (Supplementary Fig. S20A) also shows that enhanced leaf growth in myb3r1/3/5 is both due to increased cell number and increased cell size. The former mainly contributes to enhanced leaf growth during early developmental stage (Fig. 7C and D, and Supplementary Fig. S20A), whereas at later stages, enhanced leaf growth is explained mainly by increased cell size. Similar enhancement of both cell proliferation and cell enlargement was reported for the samba mutant (Eloy et al., 2012), which encodes a previously unknown activator protein of the APC/C ubiquitin ligase complex that induces degradation of mitotic proteins. Thus, the increased level of mitotic regulators, due to either enhanced transcription or reduced protein degradation, is likely the reason behind the comparable cellular and developmental phenotypes that are common to the myb3r13/5 and the samba mutants.

3) **Comment:** The observed upregulation of G2/M-specific genes could be the consequence of prolonged cell division, and not of derepression of G2/M genes (Fig. 2B, C, D - ectopic expression of G2/M transcripts in mature organs of myb3r1/3/5 mutant) (reviewer #2).

**Response:**
In Fig. 2B, we showed that CYCD3;1, which is not under the control of MYB3Rs, is downregulated in myb3r1/3/5 leaves with a similar kinetics as in wild type. Because CYCD3;1 expression is tightly linked with cell proliferation, this shows that cessation of cell division occurs more or less normally in myb3r1/3/5 leaves. In addition, our kinematic analysis of leaf growth showed that decrease in cell proliferation rate during leaf development occurred in myb3r1/3/5 with a similar kinetics to wild type. (Supplementary Fig. S20A).

4) **Comment:** Loss of myb3r1/3/5 alone enhances root growth (as in Fig. 7G and new Supplementary Fig. S20C). Therefore, observed phenotypic changes may not be due to a genetic interaction between ple-2 and myb3r1/3/5, but simply to the effect of myb3r1/3/5 mutation. (Fig. 4 - genetic interaction between myb3r1/3/5 and ple-2.) (reviewer #2).

**Response:**
Partial complementation by increased expression of mutant ple is not evident in Supplementary Fig. S14C (Supplementary Fig. S8B in old version) nor mechanistically likely. (reviewer #2)

**About genetic interaction:** The presence of genetic interaction is most clearly seen on rescuing the cellular cytokinetic defects but also evident on root growth. The data of Fig. 4A (Fig. 4B in old version) show that the cytokinesis defects in ple-2 is remarkably rescued by introducing the myb3r1/3/5 mutation. A similar genetic interaction was seen between myb3r1/3/5 and ede1-1, a weak mutant allele of EDE1, and this result was added in Fig. 4 (Fig. 4B). Moreover, the difference in root length between ple-2 and ple-2 myb1/3/5 is much more pronounced than that between wild type and myb1/3/5 (please compare Supplementary Fig. S20B and Supplementary Fig. S14A).

Therefore, it is most likely that the enhanced root growth of ple-2 when combined with myb1/3/5 reflects a true genetic interaction that can be explained by increased ple-2 transcript, not only in respect to rescuing abnormal cytokinetics, but also root growth.

**About increase of ple-2 transcript:** Supplementary Fig. S14C (Supplementary Fig. S8B in old version) showed an increase in ple-2 transcript in the myb3r1/3/5 mutant compared to wild type as determined by semi-quantitative PCR. We also showed, by real-time RT-PCR analysis of seedlings, that PLE is upregulated upon myb3r3/5 and myb3r1/3/5 mutations by about 2 fold as shown in Supplementary Fig. S3. This upregulation is reproducibly observed in our microarray analysis of seedlings and leaves (please see new Supplementary Table 1 for the list of upregulated genes in myb3r1/3/5 leaves).
About mechanisms of suppression of ple-2 phenotype: To test if increased ple-2 hypomorphic allele expression rescues the ple-2 mutant phenotype, we conducted additional experiments. We introduced the ple-2 allele under the control of CDKA;1 promoter (which drives expression mainly in proliferating cells but is not under the control of MYB3Rs) into ple-2 mutant plants. By analyzing root growth, we confirmed that transgene expression of ple-2 is able to partially complement the ple-2 phenotype (new Supplementary Fig S15A and B). We also showed that, unlike the hypomorphic ple-2 allele, the null allele of ple (pleKO caused by T-DNA insertion) was not suppressed by myb3r1/3/5 mutation (Supplementary Fig S15C), providing another piece of evidence that only the upregulation of a hypomorphic but not a non-functional null allele can rescue cytokinesis and root growth phenotypes. These genetic experiments show that MYB3R1/3/5 have functions in meristematic cells where PLE is normally expressed.

5) Comment: Loss of MYB3R3/5, which upregulates KN, should complement the cytokinesis defects in myb3r1/4, because the authors showed previously that defective cytokinesis in myb3r1/4 is mainly due to the downregulation of KN (Fig. 2A - KN expression in mutant combinations of MYB3Rs) (reviewer#2).
Response: In Fig 2B, we show that unlike EDE1, the increase of KN expression in myb3r1/3/5 mutant is pronounced in non-proliferating cells of 15 DAG leaves but non-significant in proliferating cells of 5 DAG leaves. Because Act-MYBs are only expressed in mitotic cells while Rep-MYB mutant, myb3r1/3/5, has a preferential effect to upregulate mitotic genes in non-dividing cells, we concluded that Rep-MYBs and Act-MYBs do not act in competition in the same cells. This is further verified by combining the Rep-MYB and Act-MYB mutations in the quadruple myb3r1/3/4/5 where we see distinct clusters of genes up- and down-regulated (supplementary Fig. S6). Even though the KN upregulation was cancelled out when RNA was extracted from the whole seedling and became close to wild-type level, we did not observe the rescue of cytokinesis defects as was presented in Fig. 2A (please compare D1,4 and D1,3,4,5). This lack of correlation between transcript and phenotype supports our view that the loss of Rep-MYBs affects KN expression in non-proliferating cells while Act-MYBs function in dividing cells.
We showed, in Fig. 2B, that KN and EDE1 are differently influenced by loss of Rep-MYBs. Differential effects on individual G2/M-specific genes were also observed in genome-wide scale, as in supplementary Fig. S4 and S5. We argue in the discussion, that the impact of Rep-MYBs may depend on the contexts of the target promoters, i.e. when the target promoters have strong basal activities (activities of cis elements other than MSA elements), loss of Rep-MYBs may result in promoter activation due to derepression of the basal activity, whereas such activation may not be evident when the basal activities of target promoters are low or negligible. Different contexts of target promoters may lead to different effects of Rep-MYB mutation on the transcription of distinct target genes. Such distinct regulation is also evident from our epistasis analysis with mutants in three different target genes, which show the presence (Fig. 4 for ede1 and ple) or absence (Fig. 2A for KN downregulation in myb3r1/3) of the suppression of mitotic defects when introduced into the myb3r1/3/5 background.

6) Comment: Reviewer#2 suggests additional experiments using the CYCB1;2-YFP marker at different leaf developmental stages to show cell division history in myb3r1/3/5 plants.
Response: We analyzed CYCB2;1-YFP expression in leaves at different developmental stages (7, 9, and 15 days after germination), both in wild type and myb3r1/3/5 plants. As presented in Supplementary Fig. S12, we observed increased number of YFP-expressing cells in earlier stage (7 DAG), and ectopic YFP expression in differentiated pavement cells in later stage (15 DAG). These results are consistent with our idea that myb3r1/3/5 mutation causes derepression of CYCB1;2-YFP outside the G2/M phase in proliferating cells and after cell proliferation arrest.

7) Comment: Microarray data was not analyzed sufficiently. It is necessary to provide total number and lists of deregulated genes to evaluate if they are truly G2/M phase-enriched genes, and if other cell cycle phases may not be affected (reviewer#2).
Response: We used seedlings to get insights into the genetic interactions of the myb3r mutant combinations by looking at the deregulation of a representative set of mitotic target genes (Fig. 1C-E) as well as by
microarrays to screen for gene expression changes in genome-wide scale as shown in Supplementary Fig. S4-S6. We found that the data of qRT-PCR and microarray fully matched. Due to the number of genotypes analyzed, these were single array experiments. In the analysis, we focused on the G2/M and mitotic target genes, which led us to discover that the deregulated genes are distinct in Rep-MYB and Act-MYB mutants and importantly, when the Rep-MYB and Act-MYB mutants are combined in the quadruple myb3r1/3/4/5 mutant both up- and downregulated sets of genes are present. This provides a further evidence for the model that Rep-MYB and Act-MYB mutants do not compete but work in opposition in the same cells. As suggested by the reviewer, we analyzed now these microarrays for other cell cycle genes, which confirmed that to large extent only the "mitotic" and "G2/M-specific" annotated genes are deregulated in the myb3r mutants (supplementary Fig. S5 for Rep-MYB). Based on these initial experiments on mutant combinations, we then focused on the Rep-MYB mutant, myb3r1/3/5, in two selected leaf developmental stages representing proliferating and differentiating cell populations and performed another microarray experiment, but this time with three biological replicates. This experiment allowed us to do statistical analysis to identify differentially expressed genes between wild type and myb3r1/3/5 mutant leaves (Fig. 2C, new supplementary Fig. S10, and new supplementary Table 1) as asked by the reviewer.

In the revised manuscript, we included a number of further analyses of the microarray data from both experiments (whole seedlings and mature leaves) to show the preferential effect of myb3r mutations on mitotic gene clusters.

With the whole seedling microarray, we conducted scatter plot analyses (Supplementary Fig. S5). The results show the specific upregulation of the gene sets that are annotated as “G2/M-specific” and “mitotic”, but no such effects were apparent for the gene sets related to other cell cycle phases (E2F targets, Replication, and Histone). In the gene set annotated as “core cell cycle”, upregulation is apparent only for some G2/M-specific “core cell cycle” genes (as shown by dots in cyan color in Fig. S5). These analyses clearly show that G2/M-specific genes, but not other cell cycle phases are affected by loss of Rep-MYBs, and also provide evidence that the cell cycle is not significantly affected by the myb3r mutants as discussed above.

As for the microarray data of mature leaves (microarray experiment of Fig. 2C), we conducted additional analyses and added new Supplementary Fig. S9 (scatter plot analysis), Fig. S10 (gene ontology enrichment and Venn diagram analyses), and Supplementary Table 1 (Excel file for the list of upregulated genes). Supplementary Fig. S9 again showed that, in mature leaves, myb3r1/3/5 mutation specifically affects the expression of genes annotated as “G2/M-specific” and “mitotic”, but not those related to other cell cycle phases (annotated as E2F targets, Replication, and Histones). Similarly to microarray data of whole seedlings (Supplementary Fig. S5), only G2/M-specific genes, among “core cell cycle” genes, were upregulated in myb3r1/3/5 leaves (shown by dots in cyan color in the panel of "Core cell cycle" in Supplementary Fig. S9). The Venn diagram in Supplementary Fig. S10B more clearly shows the same conclusion that the loss of Rep-MYBs specifically affects the G2/M-specific genes, but not those related to other cell cycle phases.

All the microarray data obtained in this study was deposited in GEO and currently available at the following address. http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=yruxqmqsthyxvqx&acc=GSE52298

8) Comment:
The ChIP experiment analyzing plants expressing GFP is missing. This should be done as a negative control (Fig. 5 -ChIP experiments of MYB3R3-GFP) (reviewer#2).
Response:
In the ChIP experiments, we used plants carrying the complementing MYB3R3-GFP, with an endogenous level of expression (from native promoter). The plant line that overexpresses GFP, such as the 35S::GFP with an extraordinarily large amount of GFP everywhere in the seedlings may not be a suitable control for the low level expression under native promoter in MYB3R3-GFP plants. Although we could find reports using GFP-expressing plants as negative control, non-transgenic plants are more commonly used in ChIP assays with GFP antibodies. Examples of such reports and protocols are as follows.

EMBO J. 2013, 32: 2884-2895
Proc Natl Acad Sci USA. 2011, 108: 11698-11703
Proc Natl Acad Sci USA. 2013, 110: 13192-13197
9) Comments:
The MSA motif occurs very frequently in promoters and likely also in genes that show no or an opposite response to MYB loss-of-function. Example of such genes must be included in the analysis. (Fig. 5, ChIP experiments of MYB3R3-GFP).

Response:
Fig. 5A did show the ChIP-qPCR data of IMK2, an example of a G2/M-specific gene that is not affected by Rep-MYB loss-of-function (please see qRT-PCR data in new Fig. 1C and supplementary Fig S8). This data, together with our genome-wide data of ChIP-seq analysis, clearly shows that MYB3R3 binds to the promoter of G2/M-specific genes, regardless of whether or not their expression is affected by Rep-MYB loss-of-function (Fig. 5). This notion is also consistent with our observation that sequences of MSA elements do not substantially differ between G2/M-specific genes that are strongly affected in expression by Rep-MYB mutation and those largely unaffected (Supplementary Fig. S23). As we argue in the discussion, the effect of Rep-MYB on transcription may depend on the context of target promoters (outside of the MSA elements). We discuss that the Rep-MYB-binding to MSA elements may act as repressors over the basal promoter activity (activities of cis elements other than MSA). In this line of argument, Rep-MYB may have a strong impact when the basal activity of promoters is high (as is the case for EDE7 and CYCB1;1), whereas it has little (or no) effect on target promoters with low (or negligible) basal activities (as is the case for IMK2). We are currently collecting data to prove this model through promoter-reporter gene constructs, but these experiments are outside of the scope of the present study.

Our ChIP-seq data was deposited in GEO and currently available at the following address.

10) Comment:
The size of Myb containing complex should be investigated using gel filtration to show the identity of the complex (reviewer#2).

Other homologs of DREAM subunits, at least DP, MSI1, should be also tested if they are in the same complex or not (Fig. 6, about DREAM complex) (reviewer#2).

Including other E2Fs, E2FA and E2FB, in CoIP experiments would be useful for demonstrating specificity of interaction between Myb and E2F (reviewer#3).

To test if MYB3R5 and/or MYB3R4 can bind to RBR, E2FC is another option to show the specificity of the interaction between Myb and E2F (reviewer#3).

Genetic interaction between MYB3R3 and RBR, E2FC would be helpful (reviewer#3).

Response:
We agree with the reviewers that these are interesting and important questions for a comprehensive knowledge on the plant DREAM complex. However, there are several technical limitations in plants that hinder our progress; 1) both MYB3R3-GFP and MYB3R4-GFP are low abundant transcription factors and likely to be unstable, we can only detect them in cell extracts after GFP pull down enrichment on Western blots. We also lack antibodies to DREAM complex components. Characterization of the DREAM complex through gel filtration chromatography and comprehensive determination of all the possible components and their dynamic interchange during development are not feasible with our current tools and methods.

However, because this is an important area to make progress, we performed mass spectrometry analysis of GFP pull downs with tagged lines available to us; MYB3R3-GFP, RBR-GFP and E2FB-GFP to detect associated proteins (as shown in supplementary Fig. 19). In these experiments, we could identify some of the core MuvB components of the DREAM complex (ALY2, ALY3, and TCX5) that are orthologous to the animal DREAM components (LIN9 and LIN54) in association with MYB3R3, RBR and E2FB. This is in agreement with the newly added ChIP-seq data that show the association of MYB3R3 not only with mitotic, but also with E2F target genes. Though in mass spectrometry experiments we could not detect MYB3R3 association with RBR and E2F(s), we could show these with the more sensitive ECL Western blot.

In line with the suggestions from the reviewers, we also conducted additional Co-IP experiments to uncover the specificities and developmental regulation of the MYB-RBR-E2F interactions. In these novel experiments, we found that MYB3R3 associates with RBR and E2FC, but not with E2FB (Fig. 6A and 6C). In contrast to MYB3R3, we showed that MYB3R4 (activator) associates with RBR and E2FB in early stage of leaf development with high proportion of proliferating cells (Fig. 6B), but not with E2FC at any developmental stages (Fig. 6C). This suggests that the activator and repressor MYB3Rs associate with specific E2F partners; MYB3R3 with the...
repressor E2FC and MYB3R4 with the activator E2FB. Interestingly, binding of MYB3R3 to RBR changes during leaf development suggesting that this interaction may fulfill developmental-specific functions in regulation of mitotic genes in proliferating and post-mitotic cells. These immunoprecipitation experiments and the mass spectrometry data also uncovered the likely heterogeneity and dynamic change of these complexes during leaf development.

11) **Comment:** It is unclear if CDKA;1 functions in the same complex with RBR and E2FC. Fig. 6 (DREAM complex).

**Response:**
We changed the text to avoid the confusion that led to suggest that CDKA is an integral part of the DREAM complex. CDKA is clearly a regulator as we have shown for the Act-MYBs previously (Araki et al., 2004). Therefore, it was interesting for us to test whether it can be detected with MYB3Rs.

12) **Comment:** An important control, the immunoprecipitation of the target GFP-MYB3R3 is missing Fig. 6 (DREAM complex) (reviewer#3).

**Response:**
Although it is difficult to detect MYB3R3-GFP even after GFP pull down enrichment, we do show its presence in new Figure 6A.

13) **Comment:** It is reasonable to test not only Myb target genes but also E2F target genes as well (editor and reviewer 2).

**Response:**
We conducted ChIP-seq experiment using MYB3R3-GFP plants and clearly showed that MYB3R3 binds both to the E2F targets and to canonical G2/M genes that contain MSA element. The new Fig. 5 shows enrichment of both G2/M-expressed and E2F-target genes among MYB3R3-bound genes (Fig. 5B), overrepresentation of MSA and E2F motifs in ChIP DNA (Fig. 5C), and binding of MYB3R3 around transcriptional start sites of target promoters (Fig. 5D). However, as discussed above, the myb3r1/3/5 mutation did not significantly change the expression of E2F target genes in our microarray analyses of seedlings (Supplementary Fig. S5) and leaves (Supplementary Fig. S9 and S10). The situation here is similar to that in Drosophila, where DREAM target genes containing E2F sites in their promoters are strongly affected by E2F RNAi, but not by Myb RNAi. On the contrary, DREAM targets with Myb binding sites are affected by Myb RNAi, but not by E2F RNAi (Georlette et al., 2007). Therefore, effect of each component of DREAM complex largely depends on the transcription factor that guides the complex to the targets in Drosophila, and this may be analogous in Arabidopsis. It would be interesting to examine if E2FC and/or E2FB bind to the promoters of both G2/M-specific and E2F-target genes. We would like to perform these experiments as a continuation of this work in the future.

14) **Comment:** The first half of Introduction should be rewritten, because it discusses the roles of cell proliferation in leaf development, the importance of DNA endoreduplication, and scheduled cease of cell proliferation in leaves, which, however, were not conclusively connected with the effect of rep-MYB loss-of-function in this study.

**Response:**
We substantially rewrote the entire manuscript. We briefly introduced leaf development in Introduction, because it is best understood in terms of developmental control of cell proliferation in plants. Our results showed impact of Rep-MYB on leaf development (Fig. 7C-F, and Supplementary Fig. 20A), which we analyzed quantitatively through kinematic growth analysis, as was asked.

15) **Comment:** It seems premature to define MYB3R1 as a repressor or an activator based on the number of de-regulated genes. It would be required to identify several of direct targets of MYB3R1 and investigate their expression pattern in the loss-of-function mutant.

**Response:**
We analyzed the genetic interactions in combinations of MYB3R loss-of-function mutations. Because we identified a synergistic effect on mitotic gene targets in the mutant combinations, myb3r1/3/5 (upregulation) and myb3r1/4 (downregulation), we suggested that these groups of MYBs cooperate in these functions. MYB3R1 was found to carry out both functions when combined with the respective mutants, but myb3r1 mutant on its own had little effect on mitotic gene expression (supplementary Fig. S6). The scatter plot analysis in Supplementary Fig. S5
(seedlings) and S9 (leaves) show the strong tendency for the G2/M-specific genes to be upregulated by loss of Rep-MYBs. Our previous report similarly showed the strong tendency for a cluster of mitotic genes to be downregulated by loss of Act-MYB (Haga et al., 2011). As evident from the scatter plot (Supplementary Fig. S5 and S9), the opposite effects are insignificant without exceeding general gene-to-gene variation, and probably due to secondary consequences of MYB3R mutations.

We performed additional analysis of microarray data of seedlings at 9 DAG to evaluate the effects of myb3r1 mutation under different genetic backgrounds, by comparing expression signals between wild type and myb3r1, myb3r4 and myb3r1/4, and myb3r3/5 and myb3r1/3/5 (Supplementary Fig. S7). The results show that the effects of myb3r1 mutation largely differ depending on the genetic backgrounds. In single myb3r1 mutant, the number of up- or downregulated G2/M-specific genes were small and not biased. However, introducing myb3r1 into myb3r4 or myb3r3/5 backgrounds led to a large bias on the expression of G2/M-specific genes toward downregulation in the former and upregulation in the later. No such bias was observed when all the genes were similarly analyzed. We focused on MYB3R3 to carry out ChIPseq and biochemical analysis, but the genetic synergism of myb3r1 both with myb3r4 (activator) and with myb3r3/5 (repressor), and its preferential influence on G2/M-specific genes support the dual roles of MYB3R1 for both repression and activation.

2nd Editorial Decision
09 February 2015

Thank you for submitting your revised manuscript entitled ‘Transcriptional repression by MYB3R proteins regulates plant organ growth’ as a new submission to us. Please note that I am the handling editor of your manuscript now, since Dr. Alexander Kohlmaier has left our journal. I have now received reports from all referees, which are enclosed below.

As you will see, referee #1 and #3 now support publication here, pending minor revision. Referee #2, however, raises a number of concerns that need to be addressed. Given the interest in the topic, I would like to invite you to submit a revised version of the manuscript to us, addressing all issues raised by the referees. I will not list all concerns here, as the ones raised by referee #2 are rather extensive. However, I would like to draw your attention especially to the following crucial points, which need to be completely addressed to allow publication in The EMBO Journal:

- Referee #2's concern regarding expression variations that might reduce the conclusiveness of your array data needs to be addressed for publication here.
- Referee #2's concern regarding the interaction analysis (western blots) needs to be addressed; however, we don't expect inclusion of further information on Mass-spec data (another issue raised by this referee).

Please contact me in case of other questions regarding the revision of your manuscript. Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Referee #1:

In this paper, Kobayashi et al. elucidate the function of a small group of three-repeat Myb transcription factors in Arabidopsis. This work is based on previous studies of the group showing that these Myb factor are key transcriptional regulators of mitosis. Here, the authors show that two members MYBR2 and MYBR3 act as repressors. In addition, MYBR1, which is a positive regulator of mitotic genes, was found to also act redundantly with the two inhibitors and hence appears to have a dual function. How this dual function is molecularly realized remains open at this stage. However, such an analysis goes beyond the scope of this paper and the detailed description of the repressive activity is of key importance for the understanding of the plant cell cycle.
This manuscript is an intensively revised version of a previously submitted paper. The authors have provided a substantial amount of data here and in my opinion all previously raised concerns and questions of the reviewers and the editor have been unequivocally resolved.

Referee #2:

The revised manuscript addresses a number of the concerns raised for the data in the original manuscript version. The authors have now included additional data on mutant plant development and phenotype, DNA binding of MYB complexes, and on MYB-containing protein complexes. In their response letter they also provide additional explanations for the transcription profiling experiments. However, it appears that many of the transcription profiling experiments using different mutant combinations have been performed without replicates. If correct, this information must be provided in the Methods section because the lack of biological replicates makes the data difficult to evaluate.

The conclusion that repressive MYB factors exist in plants is generally supported by the data. However, the precise function of the MYB factors and their involvement in a plant DREAM complex remains speculative and is not convincingly supported by the data. This speculation should be removed and the manuscript should be focused on actual data and their interpretation.

Although the manuscript contains a large set of interesting observations, it continues to have significant problems with selective and incomplete data presentation that makes a full evaluation of the work impossible. Transcriptome data of mutant plants without biological replicates must be regarded with great caution and they do not support strong conclusions. The protein-protein interaction data shown in the western blot is of poor quality and lack required controls. The MS/MS data are incomplete and therefore cannot be evaluated. Specific comments explaining the problems with the data and consequently the conclusions are provided below.

MYBs as activators and repressors:

The grouping of MYB3R1 as a repressive MYB is discussed in detail and supported by the data. However, there is no evidence for the activating activity of MYB3R1 (and MYB3R4) in the data. The statement in the response letter "As evident from the scatter plot (Supplementary Fig. S5 and S9), the opposite effects are insignificant without exceeding general gene-to-gene variation, and probably due to secondary consequences of MYB3R mutations" does not clarify whether or not MYB3R1 has an activating function. The authors base their claim on Fig. S6 because MYB3R1 loss of function increases gene repression by MYB3R4 (p. 8), but a similar effect can be seen in the myb3r3/4/5 mutant. Thus, the same logic suggests that the other MYBs also have an activating function. Since these data were obtained from single array experiments they have to be interpreted with caution. Consequently, the broad statement in the Introduction that "MYB3R1 and MYB3R4 act as transcriptional activators on many, if not on all, G2/M-specific genes" (p. 6) is not supported by the data and should be removed.

Mutant phenotypes:

The phenotype of mutant plants is now better documented, however, data for a detailed cell fate mapping are still missing. It appears that the mutants are quite normal, although the images in Fig. 7 suggest a larger difference in the sizes of young leaves than the graphs in Fig. S20. Also, images and descriptions of embryo phenotypes (Fig. 7I and p. 15) suggest that embryos can be quite abnormal. A similar phenotype was previously observed for MYB3R1,4 mutants by some of the same authors. Unfortunately, the authors do not provide quantification, especially for stressed plants as shown in Figure 7J. It would be important to know if the analyzed plants represent a smaller fraction of the original mutant seeds that have adapted their development (and expression programs) to the lack of the MYB factors. Such information could be obtained from the list of affected genes. The expression data for the up-regulated genes in Tab. S1 shows quite strong expression variation between replicates for some genes, particularly for the mutant plants, but also for genes with low expression in the wild-type plant. This variation is problematic for the interpretation of the other gene array
experiments that were performed without replicates, and for a comparison of fold-changes between different mutant and wild-type plants (e.g. Fig. 2B,D).

The mutant plants appear to have nearly normal cell numbers and ploidy distribution. The authors therefore argue that the mutants have normal cell cycle phase timing and cannot have prolonged G2/M phases. Based on the GFP signals of most reporter genes, however, it is quite striking that often two adjacent nuclei - probably after a recent division - express the reporters (e.g. Fig. 3). Based on the images provided, is it possible that they show karyokinesis with impaired cytokinesis? In stomata, the MYB mutation seems to have an inverse effect, i.e. it rescues cytokinesis defects of other mutants in some cases, but this might be different in other cell types. In this case, the issue of M phase length might not be answered by ploidy analysis of nuclei.

The effect of the mutations on gene expression is clear. It is shown by RT-PCR and reporter genes that some G2/M genes are up-regulated (or probably better "fail to be repressed") and this is also shown by the transcriptome data. However, the data do not allow global statements on the function of affected genes, their temporal deregulation during the cell cycle or development, or their quantitative deregulation in mutant combinations. In the absence of such information any speculative model can explain developmental effects, but this is not credible. Instead of arguing differences in timing and location of mutant effects on KN expression to explain why the cytokinesis defect of MYB3R1,4 mutants but not ede1, it would be much more convincing to show transcription effects are different in different backgrounds. The authors previously described a KN reporter, which would have been useful for such an analysis (see additional comment below).

Gene expression data:

To support their hypothesis that the analyzed MYBs act as repressors the authors focuses their discussion on genes that are up-regulated in myb3r1/3/5. The scatterplots (Fig. 2C) show that particularly at the late time point (when up-regulation of G2/M genes is most prominent) a large number of genes is also down-regulated. The manuscript does not discuss these genes although they may be of regulatory relevance. Thus, the presentation of the gene expression data in Tab. S1 is not satisfactory because it contains only up-regulated genes. The list should either contain all genes (which is very feasible in one excel table) or at least up- and down-regulated genes with a somewhat relaxed threshold. Of course, expression data will be available in GEO, but for the reviewer and readers this is an unnecessarily complicated way to access the data. All analyzed data should be provided in the context of the paper.

Roles of repressors in proliferating cells:

Using reporter gene expression the authors show that about three times more cells express certain marker genes (Fig. 3H) in the MYB mutant at early developmental stages. Should this not lead to a detectable up-regulation of these genes in the 5DAG (Fig. 2C) dataset?

As further proof for a role in proliferating cells, the authors discuss a genetic interaction with genes involved in cytokinesis and show that MYB mutation can rescue cytokinesis defects in ple and ede mutants because in double mutants the expression of deregulated genes is increased. The inclusion of ple null mutants (which are not rescued) and a CDKA:ple gene in the revised manuscript now provides more credibility for this claim, although a comparison of ple mRNA levels in the CDKA:ple and in ple/myb3r3,4 mutants would have been more convincing to demonstrate that the very moderate increase of the defective ple mRNA in the MYB mutant is indeed similar to the increase obtained with the CDKA:ple transgene. A similar cytokinesis defect, observed previously in myb3r1,4 mutants and traced to reduced KN expression, was not complemented. The authors argue (p. 11 at length in the response letter) that this is because KN is not increased in the myb3r1,3,5 mutant at early developmental times and refer to Fig. 2B. In this context, KN expression relative to wt (shown in Fig. 2B) is irrelevant and instead MYB3R1,4 and MYB3R1,3,4,5 mutants have to be compared. Fig. 1A shows that in this comparison, KN is increased to almost wild-type levels. However, since it is not mentioned at which time point the data in Fig. 1A were generated, no conclusions can be made based on the data provided.

The expression profiles of the different MYB factors are missing. This dataset would be very helpful to convince the reviewer (and support speculations) about the function of the MYB factors at
specific temporal stages of developmental. The authors state in their response letter that these MYB factors are expressed at extremely low levels such that their expression cannot be detected (???, could qRT-PCR help??), and Fig. S17 shows expression of MYB3R3-GFP.

Protein-DNA and protein-protein interactions

The authors performed ChIP-Seq experiments to address the question of whether transcriptional changes are direct effects of MYB factor mutations. The results show association with about 400 genes, many containing a MSA motif or E2F binding site. Some of these genes are up-regulated in the mutant. Similar to the transcription data, the ChIP-Seq data are not fully discussed. For example, some of the most overrepresented gene categories are DNA replication and chromatin organization/modification. It would be of interest to readers which genes were found in the GO categories and how their expression was affected. With some effort, the reviewer could deduce some of this information from Tab. S2. It is not clear why the authors do not provide a table and discussion of all the data and other features (such as presence of E2F of MSA binding sites, E2F targets, cell phase specific expression). Instead, they make only a small attempt to explain why some of the identified genes are differentially expressed in the mutant and most others are not.

Considering that they claim interactions between MYB and E2F proteins, it would have been informative to align MYB sites with lists of those E2F responsive genes which have no bona fide E2F binding site. (The gene group "E2F targets" used in the manuscript has been defined as genes deregulated by overexpression of E2F/DP and the presence of an E2F binding site. However, E2F also influences expression of many other genes).

DREAM complex

The manuscript now contains a mass-spectrometric analysis of protein complexes. Again, the authors do not provide all data and therefore they cannot be fully evaluated. Table S19 provides numbers for detected peptides for a very select few proteins. For a full evaluation a list of all detected proteins with numbers of identified peptides and their quantification must be provided with the manuscript.

Analysis by western blotting:

In Fig. 6A, one panel is not labeled. Probably that should be MYB3R3-GFP (in analogy to Fig. 6B??), although the MW would be incorrect for MYB3R3-GFP. It is disturbing that the RBR amount in the input varies strongly between the experiments in A and B, suggesting variation or irreproducibility of plant material.

The MW of E2FB also does not fit to the calculated MW (in other publications, various forms with an apparent slower migration in gel electrophoresis have been detected but also forms at the predicted MW of around 52). In this context, the band observed at 14DAG in Fig. 6A may deserve some attention and the display of a larger part of the gel should be show to also include signals in lower MW ranges.

Fig. 6C lacks a panel showing input concentrations over time for E2FC.

Of greatest concern is the absence of the immunoprecipitated GFP-MYB3R4 in the IP samples at 11 and 14 DAG (and no information on this protein in Fig. 6C), the incorrect MW of the presumed MYB3R3-GFP (if the panel in Fig. 6A should not show this protein, the information would have to be provided), and the absence of both proteins in the input fraction (Fig. 6A and B). These omissions make it impossible to evaluate the reproducibility of the data and certainly do not support discussions of temporal interaction differences.

Minor points:
The English language requires attention.

Some Figure labels are incorrect (e.g. Fig. 7D, S20A: cell area unlikely measured in "mm").
The information on members of selected gene groups (cell cycle, E2F targets, potential DREAM components) should be available in the manuscript.

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Referee #3:

This revised manuscript describes the contribution of specific MYB transcription factors to repression of G2/M genes. Initially, the investigators explored the phenotype of plants with mutations in MYB3R3 and MYB3R5 and compared with their previous studies of MYB3R1 and MYB3R4. In particular, they focused on levels of G2/M mitotic genes. Earlier work had demonstrated that MYB3R1 and MYB3R4 functioned as activators for mitotic genes. They identified redundancy in the R3/R5 double mutant to repress mitotic genes but found the phenotype stronger with the triple R1/R3/R5 mutant. A variety of assays were used to support the redundant role of MYB3R3, MYB3R5 and MYB3R1 in suppressing G2/M gene expression. Several new experiments have been added along with new analysis. Importantly, the clarity of the manuscript has significantly improved both with addition of new data as well as with clearer writing.

One interesting point is the composition of the MYB3R4 complex. In drosophila, the dREAM complex appears as a single complex containing RB and E2F homologues, the MuvB proteins, together with MYB. In contrast, the mammalian DREAM complex appears to have 2 distinct varieties, one in quiescent cells containing an RB and E2F homologue together with the MuvB proteins and a temporally distinct complex in S/G2 phase that contains MYB plus the MuvB proteins but not an RB homologue. In Figure 6, MYB3R3 was able to co-precipitate RBR1 and E2FC while MYB3R4 was able to co-precipitate RBR1 and E2FB. Furthermore, mass spectrometry data indicates that E2FB, RBR1 and MYBR3 could co-precipitate ALY2/3 and TCX5, homologues of LIN9 and LIN54 respectively. In contrast, MYB3R3 did not co-precipitate RBR1, E2FB or DPA/DPB and RBR1 or E2FB did not co-precipitate MYB3R3 (Supplementary Figure 19). These experiments suggest the possibility that composition of the various complexes may change dependent on the tissue and developmental stage.

Analysis of de novo DNA motifs from the ChIP-seq revealed E2F and MSA like motifs in Figure 5. Was there was any evidence for a CHR-like (cell cycle genes homology regions; TTTGAA) motif or other motifs enriched by MYB3R3 in the ChIP-seq analysis?

There was no mention of Supplementary Figure S7 in the main text.

Revision - authors' response 16 April 2015

Point-by-point response

We thank the three reviewers and the editor for their constructive criticism of the manuscript. We considered each of their points and revised the manuscript accordingly. Below we provide a point-by-point response to each comment.

Editor's suggestion based on the reviewers' comments:
- Referee #2's concern regarding expression variations that might reduce the conclusiveness of your array data needs to be addressed for publication here.

Our response:
Rather than performing 24 new microarray experiments, we validated the microarrays of the myb3r mutant combinations by qRT-PCR data of 9 additional mitotic genes and 1 control gene (histone H4). The mitotic genes are selected from different clusters of the microarray data showing characteristic expression patterns in mutant combinations; downregulated in Act-MYB (myb3r1/4) mutant combination (IMK2 and At1g63100), upregulated in Rep-MYB (myb3r1/3/5) mutant combination (PLE and CYCB1;2, these are from Fig. S3 of the previous manuscript), genes affected in both ways (up- and down-regulation, CDC20.1 and KN), and less strongly regulated genes (AtNACK1 and ANQ1). All the qPCR results that were done in triplicates are supporting the microarray data as shown in Supplementary Fig. S6. The qPCR expression data of EDE1 and CYCB1;1 (upregulated genes in myb3r1/3/5) shown in Fig 1D is also displayed in Fig. S6 for comparison with microarray data.
- Referee #2's concern regarding the interaction analysis (western blots) needs to be addressed; however, we don't expect inclusion of further information on Mass-spec data.

Our response:
We added further Co-IP experiments in Supplementary Fig S20, as well as added further controls on inputs (Fig. 6) and experiments on the specificity of the E2FB antibody (Supplementary Fig S20A). These Co-IP experiments are not identical repeats but confirm the dynamics and specificities of interactions between MYB3R3 with RBR and E2FC at later stages of development and MYB3R4 with RBR and E2FB in young leaves with cells actively proliferating. Additionally, we present new data that show binary interactions between MYB3R3 with the following DREAM complex components: RBR1, E2FA, E2FB, E2FC, DPA, RBR, SOL1, and MSI1. MYB3R3 was co-translation with these proteins in wheat germ extracts in vitro and we detected the binary interactions in the wheat germ extract using a method based on luminescence proximity assays, called AlphaScreen. These interaction assays also clearly show the interactions between MYB3R3 and the DREAM complex components. We also tested the interaction of MYB3R4 and the DREAM complex components, and detected interaction signal but it was much weaker. This might depend on the in vitro reconstitution of protein complexes in the wheat germ extract that is derived in primarily post-mitotic cells and the less efficient production of MYB3R4 in the wheat germ extract. The three independent approaches: 1) Pull down and mass spec identification of associated proteins, 2) co-immunoprecipitations and 3) pairwise interaction assays by AlphaScreen collectively support our conclusion for the existence of heterogeneous DREAM-like complexes in plants.

Response to the general comments from reviewer #2

1. It appears that many of the transcription profiling experiments using different mutant combinations have been performed without replicates. If correct, this information must be provided in the Methods section because the lack of biological replicates makes the data difficult to evaluate.

Our response:
As we explained clearly in our responses and in the manuscript, the array experiments in Supplementary Fig. S3 and S5 (Fig. S4 and S6 in previous version) were done with single replicate. These array experiments were aimed to discover myb3r mutant combinations that effect gene expression genome wide, such that we could use for further study including microarrays in triplicates, which we did for myb3r1/3/5 triple mutant. We are fully aware that microarray data derived from a single replicate do not allow statistical analysis, and thus to conclusively define differentially-expressed gene sets. The single array experiment allowed us to identify the mutant combinations (myb3r3/5 and myb3r1/3/5) that led to the upregulation of large cohort of mitotic genes, but not other cell cycle genes. We also found through initial microarrays that the most pronounced upregulation of mitotic genes is in older leaves with post-mitotic cells (15 DAG). We then performed triplicate microarray experiments using myb3r1/3/5 leaves at 15 DAG and statistically defined differentially-expressed genes as listed in Supplementary Table S3. Therefore, our main conclusion on repressive function of MYB3R1/3/5 is not dependent on the data of single array experiment, but on the microarray data of 15 DAG leaves performed in biological triplicate and statistically analyzed. In addition, the microarray data have been repeatedly reproduced in qRT-PCR experiments as in Fig. 1C, D, and E. Upon the suggestion of the reviewer, we expanded the qRT-PCR validation of microarray data in biological triplicates by examining 9 additional mitotic genes and 1 control genes (histone H4) using seedlings of all 8 genetic backgrounds representing myb3r mutant combinations. We designed this experiment based on the single microarray using identical conditions as shown in Supplementary Fig. S5. The results of the qRT-PCR validation are presented and compared with microarray data in new Supplementary Fig. S6. We submitted all sets of data from the microarrays and ChIP-seq experiments to GEO to make them open to the public. Upon suggestion from the Reviewer #2, we revised our manuscript by expanding preexisting or adding new Supplemental Tables (excel files) to integrate our data with other existing data sets and thus to 'facilitating readers' to gain overview of the results. We expanded Supplementary Table S2 of the previous version (list of differentially expressed genes in myb3r1/3/5) by adding many pieces of additional information on temporal expression and specific gene categories to create new Supplementary Table S3. To show the expression data of individual mitotic genes, we created new Supplementary Table S2 of a full list of G2/M-specific genes with corresponding expression data from all the microarray conducted on every mutant combination analyzed in this manuscript. The list of MYB3R3-bound genes (defined from ChIP-seq data) was
also expanded by adding quantitative expression data of myb3r mutant combinations from this (myb3r1/3/5) and our previous (myb3r1/4) studies, and many other annotations as described below in our responses to the specific comments (Supplementary Table S5). We believe that our revised version now displays the large data set more completely and effectively to the readers.

2. The precise function of the MYB factors and their involvement in a plant DREAM complex remains speculative and is not convincingly supported by the data. This speculation should be removed and the manuscript should be focused on actual data and their interpretation.

Our response:
We are disappointed by this general negative statement. In the manuscript, we analyzed myb3r mutant combinations through transcriptome, Chip-seq and detailed phenotypic analysis at the cellular and developmental levels using complementary approaches. Similarly, we used three complementary approaches to demonstrate the existence of DREAM-like complexes in plants. This wealth of data reasonably supports our conclusion on the biological roles of Rep-MYBs and of the incorporation of MYB3R3 and MYB3R4 in distinct DREAM-like complexes in plants.

3. Although the manuscript contains a large set of interesting observations, it continues to have significant problems with selective and incomplete data presentation that makes a full evaluation of the work impossible. Transcriptome data of mutant plants without biological replicates must be regarded with great caution and they do not support strong conclusions.

Our response:
See our response at points 1&2 to this repeated general negative statement.

4. The protein-protein interaction data shown in the Western blot is of poor quality and lack required controls.

Our response:
The complementing MYB3R3-GFP and GFP-MYB3R4 are expressed under the native promoters and are low abundant transcription factors, that we could not detect in crude extracts, but only when concentrated in immunoprecipitates. MYB3R3-GFP was repeatedly detected as two bands with anti-GFP antibodies, the top band corresponds to the calculated molecular weight of around 80 kD the lower 65 kD band might be a degradation product. To Fig. 6A, we added the E2FC input. We confirmed the results presented in Fig. 6 by additional Co-IP and immunoblot experiments presented in new Supplemental Fig S20B-E. As described below in our responses to the specific comments, we confirmed the specificity of E2FB antibody (Supplementary Fig S20A).

Response to the specific comments from reviewer#2

5. MYBs as activators and repressors: There is no evidence for the activating activity of MYB3R1 (and MYB3R4) in the data. The statement in the response letter "As evident from the scatter plot (Supplementary Fig. S5 and S9), the opposite effects are insignificant without exceeding general gene-to-gene variation, and probably due to secondary consequences of MYB3R mutations" does not clarify whether or not MYB3R1 has an activating function. The authors base their claim on Fig. S6 (Fig. S5 in the present version) because MYB3R1 loss of function increases gene repression by MYB3R4 (p. 8), but a similar effect can be seen in the myb3r3/4/5 mutant. Thus, the same logic suggests that the other MYBs also have an activating function. Since these data were obtained from single array experiments they have to be interpreted with caution. Consequently, the broad statement in the Introduction that "MYB3R1 and MYB3R4 act as transcriptional activators on many, if not on all, G2/M-specific genes" (p. 6) is not supported by the data and should be removed.

Our response:
We have already shown that MYB3R1 has an activating role in our previous papers (Haga et al., 2007, 2011). The statement in the Introduction (p. 6) is fully supported by our previous data that shows that combining myb3r1 with myb3r4 enhanced both downregulation of G2/M-specific genes and cytokinesis defects. The single microarray data here is consistent with our previous microarray data of myb3r1/4 (Haga et al., 2011) and the microarray experiments performed in the present work with 15 DAG leaves of myb3r1/3/5, in triplicate samples (summarized in Supplementary Table S2...
for their comparisons). Furthermore, we performed additional qRT-PCR analysis of a dozen of mitotic genes (as shown in new Supplemental Fig. S6) and confirmed gene expression changes observed in our single array experiments, especially for myb3r1, myb3r4, myb3r1/4, and myb3r3/4/5 mutant combinations. The new qPCR data shows that myb3r1, but not myb3r3/5, has significant enhancing effects on downregulation of mitotic genes (This is most prominently seen in IMK2 and At1g63100 in Fig. S6).

6. Mutant phenotypes: The phenotype of mutant plants is now better documented, however, data for a detailed cell fate mapping are still missing. It appears that the mutants are quite normal, although the images in Fig. 7 suggest a larger difference in the sizes of young leaves than the graphs in Fig. S20 (Fig. S22 in the present version). Also, images and descriptions of embryo phenotypes (Fig. 7I and p. 15) suggest that embryos can be quite abnormal.

Our response:
As the reviewer suggested, the images of Fig. 7D (seedlings at 7 DAG of wild type and myb3r1/3/5) may show larger difference of seedling growth than expected from quantified leaf size shown in Fig. S22A. We replaced these images such that they represent the difference more close to the average in leaf growth rate. As for the embryo phenotype, we repeatedly observed embryos with characteristic morphological defects and irregular division patterns in early stage (as in Fig. 7I), which, however, recovered in whole embryo morphology at later stages, and eventually produced normal-looking mature embryos as in Fig. 7B. Similar recovery of embryo morphologies has been repeatedly reported in many Arabidopsis mutants, which are regarded as evidence showing that positions of the cells in embryo, rather than division patterns, are important for pattern formation.

7. A similar phenotype was previously observed for myb3r1/4 mutants by some of the same authors. Unfortunately, the authors do not provide quantification, especially for stressed plants as shown in Figure 7J. It would be important to know if the analyzed plants represent a smaller fraction of the original mutant seeds that have adapted their development (and expression programs) to the lack of the MYB factors.

Our response:
In our observation, the generation of ectopic meristems (as in Fig. 7J) is a very rare event as we described in the text. On the other hand, the embryo phenotype is much more frequent, nearly 80% of the embryo showed morphological abnormalities in myb3r1/3/5 due to irregular and ectopic cell divisions. In the revised manuscript, we added quantitative data of the embryo defects into Supplementary Fig. S22 (Fig. S22H) and showed that myb3r1/3/5 has stronger defects than myb3r3/5, again suggesting the redundant role of MYB3R1 with MYB3R3/5 repressors.

8. The expression data for the up-regulated genes in Table S1 (Table S3 in the present version) shows quite strong expression variation between replicates for some genes, particularly for the mutant plants, but also for genes with low expression in the wild-type plant. This variation is problematic for the interpretation of the other gene array experiments that were performed without replicates, and for a comparison of fold-changes between different mutant and wild-type plants (e.g. Fig. 2B,D).

Our response:
In Table S3 (Table S1 in previous version), we performed statistical analysis using the microarray data from triplicate samples, and conclusively defined up- and down-regulated genes in the triple mutants. For the repeated mention of the single microarray see our response above.

9. The mutant plants appear to have nearly normal cell numbers and ploidy distribution. The authors therefore argue that the mutants have normal cell cycle phase timing and cannot have prolonged G2/M phases. Based on the GFP signals of most reporter genes, however, it is quite striking that often two adjacent nuclei - probably after a recent division - express the reporters (e.g. Fig. 3). Based on the images provide, is it possible that they show karyokinesis with impaired cytokinesis? In stomata, the MYB mutation seems to have an inverse effect, i.e. it rescues cytokinesis defects of other mutants in some cases, but this might be different in other cell types. In this case, the issue of M phase length might not be answered by ploidy analysis of nuclei.
Our response:

It is true that the CYCB1;2-GFP and other GFP markers showed expression in pairs of cells just after the cell division. This pattern is widely observed for the other genes related to cell division as well. In our careful observations of mutant plants, we are fully confident that there is no impaired cytokinesis, at least, in leaves, roots, cotyledons, and hypocotyls, where we observed ectopic GFP expression driven by the target promoters (as in Fig. 3, Supplementary Fig. S11 and S12). This is consistent with the absence of cytokinesis defect in stomata (Supplementary Fig. S2) and rescue in cytokinesis in both root cells (Fig 4A) and stomata (Fig. 4B). We therefore take the flow cytometry data as evidence for not having a prolonged G2 in the myb3r1/3/5 plants.

10. The effect of the mutations on gene expression is clear. It is shown by RT-PCR and reporter genes that some G2/M genes are up-regulated (or probably better "fail to be repressed") and this is also shown by the transcriptome data. However, the data do not allow global statements on the function of affected genes, their temporal deregulation during the cell cycle or development, or their quantitative deregulation in mutant combinations. In the absence of such information any speculative model can explain developmental effects, but this is not credible.

Our response:

We have already shown the basic annotations of upregulated genes in myb3r1/3/5 leaves, together with statistical analysis of expression data, in Supplemental Table S1 (Table S3 in current version). Upon the suggestion of this reviewer, we largely expanded this table and created new Supplementary Table S3 that provides a lot of additional information including our ChIP-seq results, cell-cycle phase specific expression, core cell cycle regulatory functions, and some GO categories such as "cytokinesis" and "cell cycle". We also summarized our present and previous results on a defined set of G2/M-specific genes (180 genes in total) by creating new Supplementary Table S2, which contains quantitative expression data from single microarray of every mutant combination (from Supplementary Fig. S3 and S5), statistically-analyzed microarray data of myb3r1/3/5 leaves (from Supplementary Table S3 in the present study) and our previous genome-wide expression data of myb3r1/4 seedlings (Haga e al., 2011), as well as information on responsiveness to E2F overexpression. For the gene list of each category used for analyzing large data sets (such as "G2/M-specific", "mitotic", "E2F targets", "Replication" and so on), we made new Supplementary Table S1, and provided full lists of such categories for readers.

11. Instead of arguing differences in timing and location of mutant effects on KN expression to explain why the cytokinesis defect of myb3r1/4 mutants but not ede1, it would be much more convincing to show transcription effects are different in different backgrounds. The authors previously described a KN reporter, which would have been useful for such an analysis (see additional comment below).

Our response:

We did analyzed a pKN::GUS reporter as suggested by the reviewer, but found that it did not respond to loss of Rep-MYBs as expected based on the mRNA data, probably because the KN reporter lacks some flanking sequence elements (i.e., introns or downstream regions) in addition to the upstream regions that were fused to GUS in our construct. Instead, we used GUS reporters fused to promoters of AtNACK1, CYCB1;2, and EDE1, and conclusively showed their activation in non-proliferating cells upon loss of Rep-MYBs.

12. Gene expression data: To support their hypothesis that the analyzed MYBs act as repressors the authors focuses their discussion on genes that are up-regulated in myb3r1/3/5. The scatter plots (Fig. 2C) show that particularly at the late time point (when up-regulation of G2/M genes is most prominent) a large number of genes is also down-regulated. The manuscript does not discuss these genes although they may be of regulatory relevance. Thus, the presentation of the gene expression data in Tab. S1 (Table S3 in the present version) is not satisfactory because it contains only up-regulated genes. The list should either contain all genes (which is very feasible in one excel table) or at least up- and down-regulated genes with a somewhat relaxed threshold.

Our response:

We added into Supplementary Table S1 (previous version) the significantly downregulated genes in myb3r1/3/5 and created a new Supplementary Table S3. There are only 5 such genes based on the statistical analysis we performed. The annotations of these genes are not directly associated to the
context of the mutant phenotypes, and therefore we focused our work on the large cohort of upregulated genes that are almost uniformly connected to G2/M. We made a new Supplementary Table S2, which includes the data of all G2/M-specific genes from our microarray analysis of myb3r1/3/5 leaves as well as all other microarray experiments conducted in this study.

13. Roles of repressors in proliferating cells: Using reporter gene expression, the authors show that about three times more cells express certain marker genes (Fig. 3H) in the MYB mutant at early developmental stages. Should this not lead to a detectable up-regulation of these genes in the 5DAG (Fig. 2C) dataset?

Our response:

We agree that the data in different experiments in one manuscript should be consistent with each other. We could confirm that increased number of CYCB1;2-GUS expressing cells in leaves of 9 DAG seedlings (Fig. 3H) correlated well with the upregulation of CYCB1;2 mRNA in leaves at 9 DAG plants in Fig. 2D. This figure also shows that there is no clear upregulation of CYCB1;2 in 5 DAG leaves, and this again fits with the scatter plots of the microarray data from leaves at 5DAG as shown in Fig. 2C.

14. As further proof for a role in proliferating cells, the authors discuss a genetic interaction with genes involved in cytokinesis and show that MYB mutation can rescue cytokinesis defects in ple and ede mutants because in double mutants the expression of deregulated genes is increased. The inclusion of ple null mutants (which are not rescued) and a CDKA:ple gene in the revised manuscript now provides more credibility for this claim, although a comparison of ple mRNA levels in the CDKA:ple and in ple/myb3r3.5 mutants would have been more convincing to demonstrate that the very moderate increase of the defective ple mRNA in the MYB mutant is indeed similar to the increase obtained with the CDKA:ple transgene.

Our response:

It is misleading to correlate levels of ple-2 expression and recovery of cytokinesis defects, because CDKA:1 promoter is known to be active both in proliferating cells in meristems and in cells that exited proliferation but remain competent to divide. Thus the ectopic ple-2 expression under the CDKA:1 promoter outside of the meristem is not necessarily effective for the recovery of cytokinesis defects.

15. A similar cytokinesis defect, observed previously in myb3r1.4 mutants and traced to reduced KN expression, was not complemented. The authors argue (p. 11 and at length in the response letter) that this is because KN is not increased in the myb3r1,3,5 mutant at early developmental times and refer to Fig. 2B. In this context, KN expression relative to wt (shown in Fig. 2B) is irrelevant and instead myb3r1/4 and myb3r1/3/4/5 mutants have to be compared. Fig. 2A shows that in this comparison, KN is increased to almost wild-type levels. However, since it is not mentioned at which time point the data in Fig. 2A were generated, no conclusions can be made based on the data provided.

Our response:

The data of Fig. 2A were obtained from the qPCR analysis of whole seedlings at 9 DAG. The information of the plant age was added into the legend of this figure. In addition, we have the gene expression data from myb3r1/4 and myb3r1/3/4/5 leaves as well, which showed that KN expression is decreased in young leaves of myb3r1/4. As already shown in Fig. 2B, KN is upregulated specifically in old leaves of myb3r1/3/5 mutants. As expected from a lack of complementation, in myb3r1/3/4/5 leaves, we observed both reduced KN expression in younger stage as in myb3r1/4 and increased expression in older stage as in myb3r1/3/5. On the contrary, expression of EDE1 was unaffected by myb3r1/4, and upregulated in myb3r1/3/4/5 just as in myb3r1/3/5. We added this data as Supplemental Fig S7B in the revised manuscript.

16. The expression profiles of the different MYB factors are missing. This dataset would be very helpful to convince the reviewer (and support speculations) about the function of the MYB factors at specific temporal stages of developmental. The authors state in their response letter that these MYB factors are expressed at extremely low levels such that their expression cannot be detected (???-could qRT-PCR help???), and Fig. S17 shows expression of MYB3R3-GFP.

Our response:
In Supplementary Fig. S16, we added qRT-PCR data showing expression changes of MYB3Rs during leaf development. The data shows that only MYB3R4 is confined to proliferating cells while the other MYB3Rs including MYB3R1 are expressed both in young leaves with proliferating cells and later developmental stages where most cells are differentiated. This expression pattern is fully consistent with the suggested functions as well as the dual roles of MYB3R1.

17. Protein-DNA and protein-protein interactions. Similar to the transcription data, the ChIP-Seq data are not fully discussed. For example, some of the most overrepresented gene categories are DNA replication and chromatin organization/modification. It would be of interest to readers which genes were found in the GO categories and how their expression was affected. With some effort, the reviewer could deduce some of this information from Tab. S2 (Table S4 in the present version). It is not clear why the authors do not provide a table and discussion of all the data and other features (such as presence of E2F of MSA binding sites, E2F targets, cell phase specific expression).

Our response:
As suggested by the reviewer, we included many additional pieces of information into the gene list of MYB3R3-bound genes, such as quantified gene expression changes in myb3r1/3/5 leaves (from Supplementary Table S3) and myb3r1/4 seedlings (our data from Haga et al., 2011), as well as temporal expression in the cell cycle, and gene categories (such as "E2F targets", "Replication" and some other GO categories) and created new Supplementary Table S5 (The original Table S2 with ChIP-seq data is remained as Supplementary Table S4 in revised manuscript).

18. Instead, they make only a small attempt to explain why some of the identified genes are differentially expressed in the mutant and most others are not. Considering that they claim interactions between MYB and E2F proteins, it would have been informative to align MYB sites with lists of those E2F responsive genes which have no bona fide E2F binding site. (The gene group "E2F targets" used in the manuscript has been defined as genes deregulated by overexpression of E2F/DP and the presence of an E2F binding site. However, E2F also influences expression of many other genes).

Our response:
We agree that this is a very interesting point when we consider the roles of the MYB-E2F complex and mechanisms of its action in Arabidopsis. We analyzed the overlap between E2F-responsive genes and MYB3R3-bound genes (as in new Supplementary Table S5), and found significant enrichment of E2F-responsive non-E2F target genes in MYB3R3-bound genes, suggesting the existence of some interaction between E2F and MYB3R3 for regulation of downstream genes. Functional interaction between E2Fs and MYB3Rs through mutant combinations is an important research direction we intend to take, but it is not in the scope of the present work.

19. DREAM complex. The manuscript now contains a mass-spectrometric analysis of protein complexes. Again, the authors do not provide all data and therefore they cannot be fully evaluated. Table S19 provides numbers for detected peptides for a very select few proteins. For a full evaluation a list of all detected proteins with numbers of identified peptides and their quantification must be provided with the manuscript.

Our response:
The focus of these experiments is to detect whether MYB3R3, E2FB and RBR1 make associations with DREAM-like complexes. To further emphasize and strengthen this point, we added a new table as a supplement showing the list of Arabidopsis orthologues for animal component proteins of DREAM complex (Supplementary Table S6). This should help the readers to gain an overview of the current knowledge on these evolutionary conserved proteins in plants.

20. Analysis by western blotting: In Fig. 6A, one panel is not labeled. Probably that should be MYB3R3-GFP (in analogy to Fig. 6B??), although the MW would be incorrect for MYB3R3-GFP. It is disturbing that the RBR amount in the input varies strongly between the experiments in A and B, suggesting variation or irreproducibility of plant material.

Our response:
We added the label of MYB3R3-GFP in Fig. 6A. The calculated MW for MYB3R3-GFP is 56.7 + GFP 26 = 83kDa. Repeatedly, two MYB3R3-GFP protein bands were detected with the GFP-specific antibody; a band of around 80 kDa that corresponds to the calculated MW and a faster migrating form (65kDa), the identity of which is not clear, but which could be a degradation
product. We tested the addition of MG132 proteasome inhibitor, but we could still detect the two bands (new Supplementary Fig. S20B). This shows the two forms of MYB3R3 and E2FC in the presence of MG132 and their Co-IP, while E2FB does not associate with MYB3R3. The observed difference in the developmental regulation of RBR1 protein levels in Fig. 6A and 6B could be due to the lines MYB3R3-GFP vs GFP-MYB3R4. Though these are complementing constructs, there can be increased expression for these genes, that alter RBR1 levels, e.g. by the distinct interaction dynamics of the MYB3R3 and MYB3R4 with RBR1 at late and early developmental stages, respectively.

21. The MW of E2FB also does not fit to the calculated MW (in other publications, various forms with an apparent slower migration in gel electrophoresis have been detected but also forms at the predicted MW of around 52). In this context, the band observed at 14DAG in Fig. 6A may deserve some attention and the display of a larger part of the gel should be show to also include signals in lower MW ranges.

Our response:
We are fully confident that the protein we detect with the anti-E2FB antibody that we characterized before (Plant Cell. 2005, 17: 2527-2541) is E2FB. We provide now further evidence for the specificity of E2FB antibody using mutant e2fb-1 and e2fb-1 complemented with E2FB-GFP construct, and confirmed that the antibody specifically detected the native (75 kD) and GFP-fused (110 kD) forms of E2FB (new Supplementary Fig. S20A). We performed the immunoblots on cut strips of the relevant MW regions of the gels to allow simultaneous probing with multiple antibodies.

22. Fig. 6C lacks a panel showing input concentrations over time for E2FC.
Our response:
We added the input for E2FC to Fig. 6C as requested.

23. Of greatest concern is the absence of the immunoprecipitated GFP-MYB3R4 in the IP samples at 11 and 14 DAG (and no information on this protein in Fig. 6C), the incorrect MW of the presumed MYB3R3-GFP (if the panel in Fig. 6A should not show this protein, the information would have to be provided), and the absence of both proteins in the input fraction (Fig. 6A and B). These omissions make it impossible to evaluate the reproducibility of the data and certainly do not support discussions of temporal interaction differences.

Our response:
GFP-MYB3R4 is under the control of its own promoter and as we published before (Development. 2007, 134: 1101-1110), it is only expressed in mitotic proliferating cells, and therefore not present at later leaf developmental stages at 11 and 14 DAG. This was also confirmed by analyzing transcript levels of MYB3R4 as shown in the new Supplementary Fig. S16. Both MYB3R3-GFP and GFP-MYB3R4 are undetectable in crude cell extracts (inputs) but can be detected when the MYB3R3-GFP and GFP-MYB3R4 proteins are enriched in the IP. We described in the main text and the legend that the MYB3R3-GFP and GFP-MYB3R4 are undetectable in the inputs possibly due to low expression levels from the construct with the native promoters.

Minor points:
24. The English language requires attention.
Our response:
Our original manuscript had been checked by professional proofreaders and the revised manuscript was corrected by a native English researcher.

25. Some Figure labels are incorrect (e.g. Fig. 7D, S20A: cell area unlikely measured in "mm").
Our response:
We corrected this.

26. The information on members of selected gene groups (cell cycle, E2F targets, potential DREAM components) should be available in the manuscript.
Our response:
We made a new Supplementary Table S1 showing the lists of members in all the gene groups used for Venn diagram, scatter plot, and heat map analyses in this manuscript ("G2/M-specific", "mitotic", "E2F target", "E2F responsive", "Replication", "Histone", "Core cell cycle", and...
Cell cycle phase (peak expression at G1, S, G2, and M). We also made the Supplementary Table S6 showing the list of Arabidopsis genes homologous to the component proteins of DREAM/dREAM complexes from human and fly.

Response to the comments from reviewer#1

Thank you very much for the fully supportive and constructive comments.

Response to the comments from reviewer#3

1. Analysis of de novo DNA motifs from the ChIP-seq revealed E2F and MSA like motifs in Figure 5. Was there any evidence for a CHR-like (cell cycle genes homology regions; TTTGAA) motif or other motifs enriched by MYB3R3 in the ChIP-seq analysis?

Our response:
Thank you very much for this suggestion. We also think it is very important to know if there are additional motifs enriched in our ChIP-ed fraction, because animal DREAM complex contains sequence-specific DNA binding proteins other than Myb and E2F. The CHR motif is known to be bound by Mip120/LIN54 in animals and enriched in genomic regions targeted by B-Myb and LIN9 antibodies (as shown by ChIP-seq experiment in Sadasivam et al., 2012). As shown in new Supplementary Table S6 (a list of Arabidopsis orthologues of DREAM complex components), Arabidopsis has clear orthologues of Mip120/LIN54, however we could not detect CHR-like motif in the enriched motifs in the ChIP fraction of MYB3R3-GFP. As shown in Supplementary Fig. S18D, three major motifs, other than MSA and E2F motifs, were represented as enriched sequences in MEME-ChIP analysis. However, these are not very similar to CHR motif, TTTGAA. It is very interesting to explore in future whether these enriched motifs may be the target of conserved or plant-specific component proteins of DREAM-like complex in Arabidopsis.

2. There was no mention of Supplementary Figure S7 in the main text.

Our response:
This figure was created upon the comment from the reviewer in our revision process. It shows that the effects of myb3r1 mutation on the transcriptome is dependent on the genetic background, and thus supports the dual roles of MYB3R1. However, because it was difficult to explain these results concisely within the word limits of the main text, this data was only described in our responses to the reviewers' comments. Upon the suggestion from this reviewer and from the fact that there are already 24 Supplementary figures, we decided to remove this figure from our revised manuscript.

3rd Editorial Decision 04 May 2015

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees (see comments below), and I am happy to inform you that s/he has no further objections towards publication in The EMBO Journal.

Before sending you a formal letter of acceptance, a few editorial points need to be taken care of at this stage:

- Please add to your figure legends the relevant information regarding n and error bars. This is particularly missing for figures 1 C, D, E; 2 A and D; 3 H; 7 A, C, D, E, and F as well as in the supplementary figure legends.
- Please see the attached word document, in which I suggest a few minor edits to the text in track change. Please feel free to accept or reject those and upload the revised manuscript text.
- Please check whether all figure files are of adequate resolution and quality for production, and upload improved versions if necessary.
- Please suggest (in a cover letter) 2-5 one-sentence 'bullet points', containing brief factual statements that summarize key aspects of the paper - they will form the basis for an editor-drafted 'synopsis' accompanying the online version of the article. Please see the latest research articles on our website (emboj.embopress.org) for examples - I am happy to offer further guidance on this if necessary.
As you might know, we encourage our authors to provide original source data, particularly uncropped/processed electrophoretic blots for the main figures of your manuscript. If you would like to add source data, we would welcome one PDF-file per figure for this information. These will be linked online as supplementary "Source Data" files.

I am therefore formally returning the manuscript to you for a final round of minor revision, only to allow you to easily modify/replace these files. Once we should have received them, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

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Referee #3:

The revised manuscript has been extensively re-written with the addition of new supporting data. My concerns have been adequately addressed.