molecular systems biology

Widespread inter-individual gene expression variability in *Arabidopsis thaliana*

Sandra Cortijo, Zeynep Aydin, Sebastian Ahnert and James Locke.

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Transaction Report:

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

1st Editorial Decision 18th September 2018

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your study. As you will see below, the reviewers are positive and think that the study represents a useful resource for the field. They raise however a series of concerns, which we would ask you to address in a revision.

I think that the recommendations of the reviewers are quite clear, so there is no need to repeat the points listed below. Please do not hesitate to contact me in case you would like to further discuss any of the issues raised by the reviewers. Regarding the comment of reviewer #2 referring to the lack of mechanistic insights, we do not think that delineating specific mechanisms is required for the acceptance of the study for publication. However, we would encourage you to include the gene regulatory network analysis suggested by reviewer #2, to further examine the potential contribution of TFs to the observed variation.

REFEREE REPORTS

Reviewer #1:

This manuscript explores variation in gene expression among individual Arabidopsis plants. This is an interesting topic because there is a growing awareness that even among genetically identical individuals there can be considerable variation in gene expression. Ultimately this may lead to a better understanding of stochasticity in phenotype and in environmental effects on development, disease, and morbidity. While this topic has received significant attention in microbes, there are few studies in plants and this explores the characteristics of genes with high or low variability in expression in far more detail than other studies that I am aware of. While mostly descriptive and correlative, this works serves as an excellent foundation for future studies by its in depth analyses. Writing is very clear, conclusions are justified.

Major points:

1. The authors define a set of highly variable genes (HVG) and then compare this to a set of random genes of similar size. The general standard when this type of empirical control is used is to create many random gene sets (100 or 1000) and then compare the test set (HVG in this case) to the distributional properties of the random sets. This allows statistical conclusions to be drawn about the differences between the test set (HVG) and random expectation. Shouldn't this approach be taken here?

2. Some important references are missing. The statement "gene expression variability has only been analysed for a few individual genes in plants" is incorrect. For example, Dan Kliebenstein's lab has also explored variation in Arabidopsis gene expression. The correct paper is actually cited (Jimenez-Gomez et al, 2011) but only in the context of phenotypic variance, not genome-wide expression variation. The prior work should be properly acknowledged and current results compared to previous findings. The Jimenez-Gomez paper has a very different focus than this manuscript so the prior work does not diminish the impact of the current manuscript. Similarly, Lin et al (G3, 2016) have explored these questions in Drosophila. This work should also be discussed and results compared to the current findings. I did not do an exhaustive literature search, but since these papers were missed I would encourage the authors to explore the literature and make sure there are not papers beyond these two that are relevant.

Minor points:

line 181: typo "is higher that between"

Fig 2C, S3C, S3D. The same cutoffs for heatmap shading should be used in the figures so that it is easy to compare. One approach would be to base this on p-value (or -log10(p) for significant overlap based on Fisher's exact test.

Fig S4. Need information on how tissue specificity was ascertained. What data set? What analysis?

line 277: The 0.4 and -.4 cutoffs seem rather arbitrary. Justify. Or better, use a significance cutoff instead.

Figure 4: x-axis labels should match what is being used in the text: HVG, LVG, random.

lines 290-295. A statistical approach to the gene length vs # of introns question would be useful. which has a larger R2 in linear regression? If you start with intron# as the explanatory variable is a better fit obtained when gene length is added? how about the other way around?

Figure S1 legend. "pearson" should be capitalized (multiple occurences).

Reviewer #2:

The manuscript by Cortijo and colleagues describes a transcriptomics resource containing 168 datasets derived from profiling 14 individual Arabidopsis seedlings at 12 time points over the course of one day. The manuscript delves into the analysis of gene expression variation and a number of interesting observations are presented. Highly variable genes of several classes are identified and their function and regulation are analysed. Some of the tale home messages are that variable genes tend to be associated with functions in environmental response and that these genes are on average shorter and embedded in more repressive chromatin environments compared to random genes.

The data and analysis presented is very solid and represents a beautiful resource for the community, however it falls a bit short of my expectation when it comes to elucidating the mechanisms behind the observed gene expression variation. The authors identify 60 transcription factors enriched upstream of highly variable genes, but do not analyse their potential contribution to variation. The

claim that it is unlikely that variation is encoded at the TF level, because only seven of them are variable themselves, is weak, since a single variable input into a highly connected network with many nodes can result in global variation. The authors should therefore attempt to reconstruct a gene regulatory network from the HVGs and their transcription factors to analyses this aspect more rigorously.

Minor points: The manuscript is somewhat difficult to read here and there since the authors like to use the term "detected" in a number of contexts. To me, in the context of gene expression profiling "detected" stands for evidence of expression more than anything else.

Reviewer #3:

In this manuscript Cortijo and colleagues describe a new transcriptomics resource for A. thaliana. The authors have generated RNA-seq profiles for 168 plant seedlings at 12 time points during a 24h period. The originality of their approach is the high number of replicates (14) analysed at each time-points. This permitted to calculate a variability score for each gene at each time-points providing insight into the levels and the dymanics of gene expression variability during the day. Importantly this resource is made available to the wider community through a web interface. To showcase their dataset the authors defined HVGs at each time-points and found that these were involved preferentially in response to external conditions, were short, had many TF binding-sites and had a repressive chromatin architecture (based on published data).

This is a well written paper describing a great resource. The web interface set up by the authors is a very significant strength of this work which will ensures easy access by the community. I have a few comments and suggestions, which I hope will help improve the manuscript.

Major:

1) I am not familiar with A. thaliana biology and life cycle as it may be the case of many readers. In order to provide an accurate description their biological system, I think the authors should include on figure 1 a real picture of plants grown in conditions identical to those used in the paper. This would help the reader to understand the extent of phenotypic diversity present in the samples and to get a feeling about how similar each plant environment is on the plate.

2) The authors talk about gene expression variability between seedlings. This variability is apparent after averaging expression levels over thousands of cells as each RNA-seq library was made from a single whole organism. It would be useful if the authors could elaborate a bit in their introduction about what this form of variability really is. It clearly is not the sort of noise observed between single prokaryotic cells for instance. Related to the point above, do the authors assume that the 14 seedlings were grown in identical environments? For instance are seedling growing at the edges of the plate different than those from the middle?

3) Moreover, how many cell types are there in these seedlings? Could the relative proportion of different cell types participate to the observed variability?

4) On Figure 3E, the mean normalised expression ranges from 0.8 to 1.2, while on Figure 1B the average read counts span two orders of magnitude. I have not done the maths but this seems strange. Why have the authors chosen to compress the colour bar so much on Figure 3E? Aren't we missing some information here?

5) On Figure S5B, I really think the authors have to investigate the bimodal distribution of HVGs correlations with expression levels further. By this I mean consider the genes from each two peaks as a different group. Are those with high CV-mean correlations specifically induced during the day or the night for instance? Are those from the peak with low correlation lower of higher expressed? Etc...

6) Regarding the analysis of histone modifications. I think the authors should mention at the beginning of the paragraph how their ChIP-seq data compare to their time course in term of

experimental conditions. Where they acquired during the day? Or during the night?

7) Moreover, do the authors think that HVGs are expressed despite their repressive context? Or rather, that their chromatin structure is also variable (which would have been averaged out in the published ChIP-seq data)?

Minor:

1) Figure 3C, please provide the p-values for the red and green dots.

2) Authors find that HVGs tend to be short. Are short genes associated with any specific GO category in A. thaliana? If stress response genes were to be, it could explain this enrichment.

3) Line 405, "similar but different" mechanisms of generation of transcriptional variability. If I get this correctly, regulation at the level of one genome in individual cells is compared to a form of coordinate response of many cells at the organism level. What is "similar"? Do the authors think that some seedling are more responsive than others? Or rather that some are experiencing higher levels of stress?

We thank the reviewers for their positive appreciation of the manuscript and very useful comments and suggestions. We have included results and corrections as recommended by the reviewers, which we believe have significantly improved the manuscript. A detailed response to all comments can be found below.

Reviewer #1:

This manuscript explores variation in gene expression among individual Arabidopsis plants. This is an interesting topic because there is a growing awareness that even among genetically identical individuals there can be considerable variation in gene expression. Ultimately this may lead to a better understanding of stochasticity in phenotype and in environmental effects on development, disease, and morbidity. While this topic has received significant attention in microbes, there are few studies in plants and this explores the characteristics of genes with high or low variability in expression in far more detail than other studies that I am aware of. While mostly descriptive and correlative, this works serves as an excellent foundation for future studies by its in depth analyses. Writing is very clear, conclusions are justified.

Major points:

1. The authors define a set of highly variable genes (HVG) and then compare this to a set of random genes of similar size. The general standard when this type of empirical control is used is to create many random gene sets (100 or 1000) and then compare the test set (HVG in this case) to the distributional properties of the random sets. This allows statistical conclusions to be drawn about the differences between the test set (HVG) and random expectation. Shouldn't this approach be taken here?
Thank you for this suggestion. We have now created 1000 sets of random genes of the same size of the HVGs to compare with HVGs for the distributions of the corrected CV^2, the number of time-points where genes are selected, gene length, number of introns, number of TFs targeting each gene, gene expression tissue specificity (entropy), gene expression level and for the correlation between profiles in gene expression and variability. The average and 95% interval calculated from these 1000 sets are now used in the corresponding figures.

2. Some important references are missing. The statement "gene expression variability has only been analysed for a few individual genes in plants" is incorrect. For example, Dan Kliebenstein's lab has also explored variation in Arabidopsis gene expression. The correct paper is actually cited (Jimenez-Gomez et al, 2011) but only in the context of phenotypic variance, not genome-wide expression variation. The prior work should be properly acknowledged and current results compared to previous findings. The Jimenez-Gomez paper has a very different focus than this manuscript so the prior work does not diminish the impact of the current manuscript. Similarly, Lin et al (G3, 2016) have explored these questions in Drosophila. This work should also be discussed and results compared to the current findings. I did not do an exhaustive literature search, but since these papers were missed I would encourage the authors to explore the literature and make sure there are not papers beyond these two that are relevant.
Thanks for this comment. We have now included these references (and others) in the

introduction and also described more the meaning of inter-individual transcriptional variability.

Minor points:

line 181: typo "is higher that between"
Corrected.

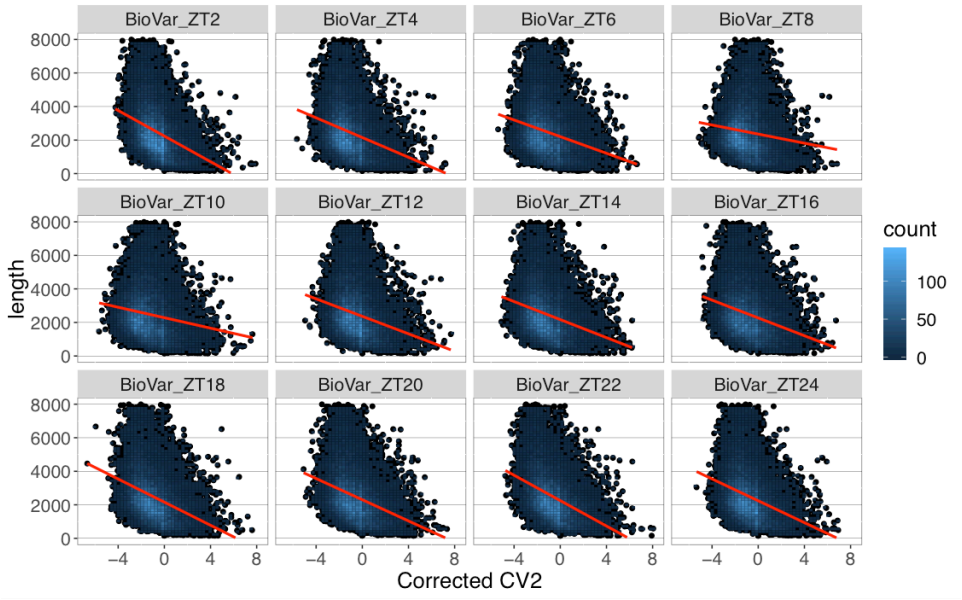
Fig 2C, S3C, S3D. The same cutoffs for heatmap shading should be used in the figures so that it is easy to compare. One approach would be to base this on p-value (or -log10(p) for significant overlap based on Fisher's exact test.
Thanks for this suggestion. We have now added Appendix Fig S3G that includes heatmaps for HVGs, LVGs and one set of random genes using the same cutoffs of the heatmap shading.

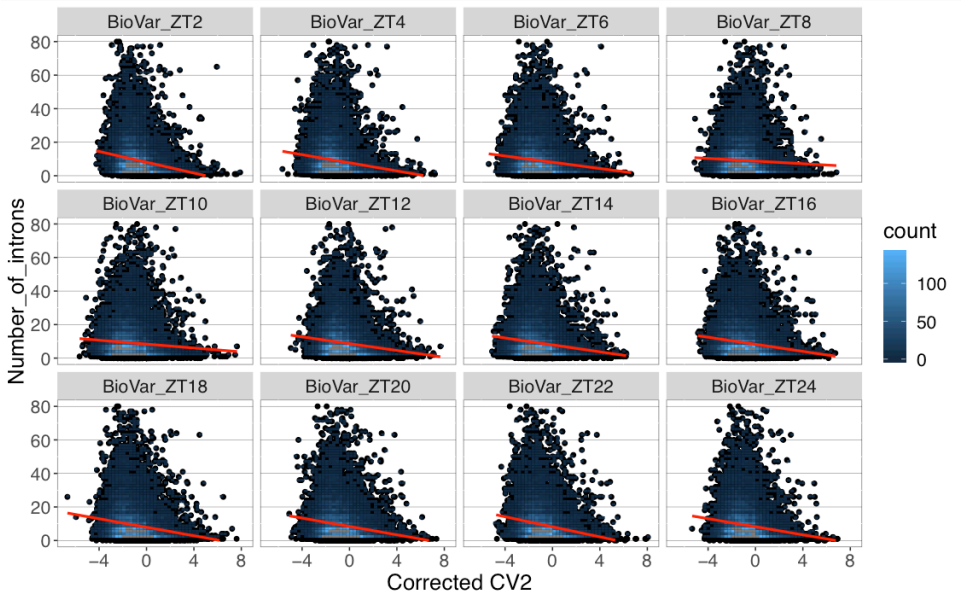
Fig S4. Need information on how tissue specificity was ascertained. What data set? What analysis?
Thanks for this comment. We have added the following information in the material and methods section:
L623 "Shannon entropy from Roudier and colleagues (Roudier et al., 2011) was used to measure gene expression tissue specificity of HVGs, LVGs and the thousand sets of random genes. It was calculated using publicly available developmental expression series (Schmid et al., 2005), after filtering genes that showed no expression in any conditions."

line 277: The 0.4 and -.4 cutoffs seem rather arbitrary. Justify. Or better, use a significance cutoff instead.
Thanks for this suggestion. We now use a significance cutoff of p-value less or equal to 0.05 and find 285 HVGs with a significant correlation between profiles of expression levels and variability (20% of all 1358 HVGs). We changed this in the manuscript.
L304: "If we consider HVGs with a significant correlation (p-value less or equal to 0.05), it seems that profiles in gene expression variability for approximately 20% of HVGs could be potentially explained by expression profiles (for profiles of positive and negative correlations see examples in Appendix Fig S5C-D)."

Figure 4: x-axis labels should match what is being used in the text: HVG, LVG, random.
Figure 4 was changed accordingly to major point 1, which also solves this point.

lines 290-295. A statistical approach to the gene length vs # of introns question would be useful. which has a larger R2 in linear regression? If you start with intron# as the explanatory variable is a better fit obtained when gene length is added? how about the other way around?
Thanks for this suggestion. We have now performed this analysis and unfortunately a linear regression is not the best way to capture the relation between the $\log_2(CV^2/\text{trend})$ and the gene length or number of introns, as you can see in the figures below. This can also explain the low R^2 values in the table below. We thus decided to not include these results in the manuscript.





Time point	R2 of the linear fit CorCV2~number of intron	R2 of the linear fit CorCV2~gene length	R2 of the linear fit CorCV2~number of intron + gene length
ZT2	0.036	0.108	0.109
ZT4	0.021	0.060	0.060
ZT6	0.014	0.048	0.049
ZT8	0.001	0.004	0.005
ZT10	0.004	0.013	0.013
ZT12	0.015	0.045	0.046
ZT14	0.017	0.058	0.059
ZT16	0.019	0.062	0.064
ZT18	0.028	0.111	0.116
ZT20	0.028	0.091	0.093
ZT22	0.041	0.136	0.139
ZT24	0.022	0.085	0.088

Figure S1 legend. "pearson" should be capitalized (multiple occurences).
Thanks for this remark; this has been changed throughout the manuscript.

Reviewer #2:

The manuscript by Cortijo and colleagues describes a transcriptomics resource containing 168 datasets derived from profiling 14 individual Arabidopsis seedlings at 12 time points over the course of one day. The manuscript delves into the analysis of gene expression variation and a number of interesting observations are presented. Highly variable genes of several classes are identified and their function and regulation are analysed. Some of the tale home messages are that variable genes tend to be associated with functions in environmental response and that these genes are on average shorter and embedded in more repressive chromatin environments compared to random genes.

The data and analysis presented is very solid and represents a beautiful resource for the community, however it falls a bit short of my expectation when it comes to elucidating the mechanisms behind the observed gene expression variation. The authors identify 60 transcription factors enriched upstream of highly variable genes, but do not analyse their potential contribution to variation. The claim that it is unlikely that variation is encoded at the TF level, because only seven of them are variable themselves, is weak, since a single variable input into a highly connected network with many nodes can result in global variation. The authors should therefore attempt to reconstruct a gene regulatory network from the HVGs and their transcription factors to analyses this aspect more rigorously.
Thanks for this suggestion. We have now analysed more in detail the regulation of HVGs by the 60 TFs with targets enriched in HVGs, of which 7 TFs are themselves highly variable. As suggested, we also derived gene regulatory networks for HVGs and these TFs based on the DAP-seq data. We added the following in the results:
L251: “1106 out of 1358 HVGs are potential targets of at least one of these 7 TFs. However 23301 genes in total are potential targets of at least one of these 7 TFs, so only a small fraction of these potential targets are HVGs (Table EV5). Moreover, DAP-seq data being derived from in vitro interaction provides a list of potential targets and further experiments such as ChIP-seq would be required to obtain the list of genes regulated by these TFs in our conditions. When deriving gene regulatory networks from the DAP-seq data for HVGs and these TFs, we observe a high level of regulation of these 60 TFs by other TFs of this same list, and that most HVGs are targeted by a combination of highly variable and non-highly variable TFs (Appendix Fig S4C-E and Table EV6). These results suggest that while the high level of variability could potentially partly be explained by TFs, other factors are also probably involved.”

However, we have to keep in mind that DAP-seq is performed in vitro and is thus only providing a list of potential targets. We do not know what is the proportion of these potential targets that are actually regulated by the TFs. Other experiments, such as ChIP-seq and RNA-seq of mutants for these TFs, would be required to refine these gene regulatory networks.

We would also like to add that we are currently performing a network analysis of the transcriptomic dataset of this manuscript, which will be the main focus of a paper under preparation.

Minor points: The manuscript is somewhat difficult to read here and there since the authors like to use the term "detected" in a number of contexts. To me, in the context of gene expression profiling "detected" stands for evidence of expression more than anything else.
Thanks for this remark; this has been changed throughout the manuscript.

Reviewer #3:

In this manuscript Cortijo and colleagues describe a new transcriptomics resource for A. thaliana. The authors have generated RNA-seq profiles for 168 plant seedlings at 12 time points during a 24h period. The originality of their approach is the high number of replicates (14) analysed at each time-points. This permitted to calculate a variability score for each gene at each time-points providing insight into the levels and the dymanics of gene expression variability during the day. Importantly this resource is made available to the wider community through a web interface. To showcase their dataset the authors defined HVGs at each time-points and found that these were involved preferentially in response to external conditions, were short, had many TF binding-sites and had a repressive chromatin architecture (based on published data).

This is a well written paper describing a great resource. The web interface set up by the authors is a very significant strength of this work which will ensures easy access by the community. I have a few comments and suggestions, which I hope will help improve the manuscript.

Major:

1) I am not familiar with A. thaliana biology and life cycle as it may be the case of many readers. In order to provide an accurate description their biological system, I think the authors should include on figure 1 a real picture of plants grown in conditions identical to those used in the paper. This would help the reader to understand the extent of phenotypic diversity present in the samples and to get a feeling about how similar each plant environment is on the plate.
Thanks for this suggestion. We grew plants under identical conditions to those used in our experiments and took a picture that is now in Appendix Fig S1A.

2) The authors talk about gene expression variability between seedlings. This variability is apparent after averaging expression levels over thousands of cells as each RNA-seq library was made from a single whole organism. It would be useful if the authors could elaborate a bit in their introduction about what this form of variability really is. It clearly is not the sort of noise observed between single prokaryotic cells for instance. Related to the point above, do the authors assume that the 14 seedlings were grown in identical environments? For instance are seedling growing at the edges of the plate different than those from the middle?
Thanks for this comment. We have now included the following sentences in the introduction and discussion of the manuscript to answer this point:

L55: “It is not known if such inter-individual phenotypic variability originates from responses to microenvironmental perturbations or from stochastic factors at the cellular level, or from both.”
L445: “Moreover, we do not know if HVGs exhibit similar behaviour in different parts of the plant, or whether these genes are more or less variable in different parts of the plant. Further analysis of inter-individual gene expression variability in different tissues (e.g in roots, hypocotyls, aerial parts etc…) would be required to answer this question.”

Moreover precautions were taken during the experiment to reduce developmental and environmental variability. As explained in the material and methods, we could not see a plate effect when analysing gene expression.
L524: “In order to reduce environment effects, all seedlings harvested for one time-point were growing in the same plate, and seedlings that looked smaller than others were not harvested. Moreover, the seedling number corresponds to the seedling position in the plate and we could not see any obvious position effect when analysing gene expression variability (Appendix Fig S2G). Only seedlings for which the root was on the surface of the MS media were harvested, in order to avoid breaking roots while harvesting.”

We now also included heatmaps of the hierarchical clustering, for each time-point, of HVGs and of individual seedling using the mean normalised expression levels, further showing that seedlings at the edged of the plate do not show similar expression profiles (Appendix Fig S2G).

3) Moreover, how many cell types are there in these seedlings? Could the relative proportion of different cell types participate to the observed variability?
The first point is a very interesting question to which there is no consensus. We can only say that an Arabidopsis seedling contains a high number of different cell types. About the second point, we now analysed the expression level of the HVGs in the different tissues using the same dataset we used for the entropy analysis. We find that most HVGs are expressed in more than one tissue suggesting that the relative proportion of different cell types would not be the primary cause of the observed variability (Appendix Fig S4B).

4) On Figure 3E, the mean normalised expression ranges from 0.8 to 1.2, while on Figure 1B the average read counts span two orders of magnitude. I have not done the maths but this seems strange. Why have the authors chosen to compress the colour bar so much on Figure 3E? Aren't we missing some information here?
Thanks for this remark. On Fig 3E gene expression for each gene is normalised by its average expression level across the time-course. This is not normalised by the average of all genes in one time-point and thus cannot be directly compared with Fig 1B. We have added more information on the material and section to avoid any confusion.
L591: “Mean normalised gene expression was used when representing gene expression throughout the time course. It was calculated for each gene by dividing the expression level at a given time-point by the average expression across the entire time course for the same gene.”

5) On Figure S5B, I really think the authors have to investigate the bimodal distribution of HVGs correlations with expression levels further. By this I mean

consider the genes from each two peaks as a different group. Are those with high CV-mean correlations specifically induced during the day or the night for instance? Are those from the peak with low correlation lower of higher expressed? Etc...
Thanks for this comment. We have now explored the HVGs under the two peaks and compared their profiles in expression, their expression levels and the number of time points when they are identified as highly variable. We could only find a difference for the expression level, for which HVGs with a positive correlation between variability and expression levels have a lower expression level in general compared to other HVGs. We have integrated these results in the manuscript:
L300: "We cannot see major differences in the expression profiles or the number of time-points for which genes are identified as HVGs for these two groups of genes (Appendix Fig S5C-D). However, HVGs with a positive correlation between variability and expression levels (peak around 0.5 in Appendix Fig S5B) have a lower expression level in general compared to other HVGs (Appendix Fig S5E)."

6) Regarding the analysis of histone modifications. I think the authors should mention at the beginning of the paragraph how their ChIP-seq data compare to their time course in term of experimental conditions. Where they acquired during the day? Or during the night?
Thanks for this question. ChIP-seq data are extracted from available datasets and we unfortunately don't have information about the time of the day at which the plants were harvested to perform the ChIP-seq. The following has been added in the manuscript to clarify this point:
L356: "we analysed several histone marks using data already available for which we have no information about the time of day when the plants where harvested."

7) Moreover, do the authors think that HVGs are expressed despite their repressive context? Or rather, that their chromatin structure is also variable (which would have been averaged out in the published ChIP-seq data)?
This is an interesting point. Correlation between expression variability and chromatin state has also been observed in previous studies in other organism and we do not know yet if, in one cell, a gene is expressed while despite a repressive environment. We added the following in the discussion to reflect this point:
L489: "The ChIP-seq data we used were obtained from bulk plant experiments, but in the future it would be of interest to directly compare chromatin marks and expression levels by performing RNA-seq and ChIP-seq or BS-seq on the same individual seedling or cell. Although very challenging, recent advances on single-cell RNA-seq, ChIP-seq and BS-seq indicate that such types of experiment could be possible. Variability in DNA methylation was for example recently reported using single-cell approaches in human (Ecker et al., 2017; Garg et al., 2018)."

Minor:

1) Figure 3C, please provide the p-values for the red and green dots.
Thanks for this suggestion. p-values of the Fisher's exact test have been added to the Fig 3C.

2) Authors find that HVGs tend to be short. Are short genes associated with any specific GO category in A. thaliana? If stress response genes were to be, it could explain this enrichment.

Thanks. This in indeed the case, and this point is included in the discussion of the paper:
L471: “It has also been shown in *Arabidopsis thaliana* that stress responsive genes are shorter (Aceituno et al., 2008), in agreement with the fact that HVGs are enriched in environmentally responsive genes.”

3) Line 405, "similar but different" mechanisms of generation of transcriptional variability. If I get this correctly, regulation at the level of one genome in individual cells is compared to a form of coordinate response of many cells at the organism level. What is "similar"? Do the authors think that some seedling are more responsive than others? Or rather that some are experiencing higher levels of stress?
Thanks for this comment. The following sentence was included in the discussion of the manuscript to take into account this point:
L442: “It would be of interest to define if similar types of gene regulatory circuits are involved in the generation of this transcriptional variability, and whether it could also originate from variability in the stress level of seedlings or their responsiveness to the environment.”

2nd Editorial Decision 6th December 2018

Thank you for sending us your revised manuscript. We are satisfied with the modifications made and we think that the study is now suitable for publication.

Before we formally accept the study for publication, we would ask you to address the following minor editorial issues:

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: James Locke
Journal Submitted to: Molecular Systems Biology
Manuscript Number: MSB-18-8591

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Page 24, Line 563: "Samples with at least 4 million reads were used, which is between 14 and 16 samples per time-point (Table EV1). To define the number of seedlings to use in order to identify transcriptional variability, we compared the corrected square coefficient of variation (corrected CV2) obtained when analysing 6 to 15 seedlings with the ones obtained with 16 seedlings at the time point ZT6, as we collected up to 16 seedlings for this time-point (Appendix Fig S1D). We observed a plateau in the increase of correlation from 10 or more seedlings, with a correlation of more than 0.9 between the corrected CV2 calculated using 16 seedlings and the ones calculated with a least 12 seedlings (Appendix Fig S1D). As our dataset contains 14 to 16 seedlings for each time-point, we thus decided to use 14 seedlings in all cases to be able to compare the time-points. When more than 14 seedlings were available for one time-point, we removed the extra seedlings with the lowest number of reads. This is higher than what was done in plants until now, as Folta and colleagues (Folta et al., 2014) analysed inter-individual expression variability for 8 genes using 6 seedlings, and Brennecke and colleagues (Brennecke et al., 2013) analysed gene expression variability using scRNA-seq for 7 cells."
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Page 24, Line 563:"Samples with at least 4 million reads were used, which is between 14 and 16 samples per time-point (Table EV1). "
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Page 22, Line 524:"In order to reduce environment effects, all seedlings harvested for one time-point were growing in the same plate, and seedlings that looked smaller than others were not harvested. Moreover, the seedling number corresponds to the seedling position in the plate and we could not see any obvious position effect when analysing gene expression variability (Appendix Fig S2G). Only seedlings for which the root was on the surface of the MS media were harvested, in order to avoid breaking roots while harvesting. "
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, see legends of figures, material and method section and in results

Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistical tests used are: Chi-square test, Spearman correlation and Wilcoxon text. There is no assumption about normality of the data for these tests. Sample size is high enough for the Chi-square test.
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Page 27, line 649: "RNA-seq data: Gene Expression Omnibus GSE115583 (link will be included once the paper is accepted)."
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Page 27, line 649: "RNA-seq data: Gene Expression Omnibus GSE115583 (link will be included once the paper is accepted)."
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Page 27, Line 654:"Computer codes used to analyse RNA-seq and ChIP-seq data: GitHub (link will be included once the paper is accepted)"

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA