molecular systems biology

Systems-based analysis of Arabidopsis leaf growth reveals adaptation to water deficit

Katja Baerenfaller, Catherine Massonnet, Sean Walsh, Sacha Baginsky, Peter Bühlmann, Lars Hennig, Matthias Hirsch-Hoffmann, Katharine A. Howell, Sabine Kahlau, Amandine Radziejwoski, Doris Russenberger, Dorothea Rutishauser, Ian Small, Daniel Stekhoven, Ronan Sulpice, Julia Svozil, Nathalie Wuyts, Mark Stitt, Pierre Hilson, Christine Granier, and Wilhelm Gruissem,

Corresponding author: Katja Baerenfaller, ETH Zurich

Review timeline:

Submission date:	07 April 2012
Editorial Decision:	21 May 2012
Revision received:	19 June 2012
Editorial Decision:	12 July 2012
Revision received:	17 July 2012
Accepted:	25 July 2012

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 21 May 2012

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 manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise, however, substantial concerns on your work, which, I am afraid to say, preclude its publication in its present form.

Overall, the reviewers felt that the data provided here may be of use to the plant biology community, and they found some of the new observations potentially intriguing, particularly the relative lack of day/night oscillations in protein levels. Nonetheless, they felt that additional work was needed to rigorously demonstrate that this represents a genuine biological difference in mRNA vs protein variability, rather than a technical lack of power to detect protein abundance changes (due to either iTRAQ underestimation of protein abundance changes, or a bias toward detection of high-abundance proteins). Given that this represents one the most novel biological results of this work, the editor feels that this issue will need to be conclusively addressed in any revised work. The last reviewer felt that additional validating experiments with an orthogonal technology (e.g. Western Blots and qRT-PCR) would be absolutely needed to support the existing transcriptomic and proteomic data. The second reviewer raised a series of similar issues, and was clearly troubled enough by these points that s/he rated the "validity of conclusions" as "low" within our online submission system, further stressing the need for additional investigation and supporting evidence.

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of

the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

When submitting your revised work please also consider the standard revision checklist (below), and please include individual image files for each figure.

Please note As part of EMBO Publications' Transparent Editorial Process, Molecular Systems Biology now publishes a Review Process File with each accepted manuscript. In the event of acceptance, your cover letter/point-by-point response will be included in this file, which will be available to the scientific community. Authors may opt out of the transparent process at any stage prior to publication (contact us at msb@embo.org). More information is available in our Instructions to Authors (http://tinyurl.com/79zy6ap).

Sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Referee reports:

Reviewer #1 (Remarks to the Author):

Baerenfaller et al. report the first systems-level analysis of leaf growth, transcript accumulation and protein abundance study for leaf 6 of Arabidopsis thaliana. Strategic consideration of plasticity associated with the developmental by environmental interaction was incorporated into the experimental design by including a comparative examination of transcript and protein accumulation at four carefully defined developmental stages, two points in the diurnal cycle, and two growth conditions. Despite the enormous complexity of the dataset, the presentation is highly engaging. Not too surprisingly, the authors have provided support for a number of earlier observations as well as many first findings. This report and its accompanying dataset will be of considerable interest to plant biologists.

Small details:
1) Page 5, second paragraph, last sentence needs addition of the word, 'for' in "could not compensate 'for'..."

2) Figure 5: This is a very important summuative figure, but unfortunately it is extremely difficult to decode. First, the size of the figure needs to be large enough for the reader to see the distinction in color and the labeling. The legend does not provide sufficient support. The authors have presented their code for differential mRNA and protein accumulation in the main text; some of this information ought to be repeated in the legend. The legend should include definitions for abbreviations. Perhaps colored font can be used to help aid interpretation, such as a different colored font can be added. Can information for the number of genes in each branch be included? The red and blue color that is supposed to be evident does not come across.

Reviewer #2 (Remarks to the Author):

Baerenfaller and colleagues present a comprehensive analysis of leaf characteristics across developmental time in plants grown under optimal and mild water stress conditions. They carefully analyzed growth parameters of the first adult rosette leaf (leaf #6) to define four distinct growth stages. They found that the water limiting conditions slowed the pace of growth and resulted in smaller plants with fewer cells and reduced polyploidy. For genomic analyses, they defined four developmental stages and collected samples of plants grown in both water conditions at the end of the night (EON) and end of the day (EOD). Importantly, the same biological samples were used for transcriptomic (using a tiling array) and proteomic analysis (using iTRAQ). The authors clustered the protein and transcript data to find distinct patterns in accumulation and compare these between

the two growth conditions.

In their analysis of transcript levels, the authors report large differences across the developmental and time-of-day samples. They also find that the water-limited plants show reduced amplitude of oscillations in gene expression compared to the plants grown in optimal water conditions.

In contrast, they find that very few proteins show differences in abundance across their time-of-day samples. The iTRAQ technique has limited sensitivity, allowing only a small fraction of the proteome to be quantifiably assessed in these experiments (2081 and 1509 proteins in the water optimal and water limiting conditions, respectively). In addition, iTRAQ has previously been reported to underestimate actual changes in protein levels (Pierce et al, Molecular and Cellular Proteomics, 2008).

Of the proteins that could be quantified, almost none showed daily changes in abundance and only ~25% showed changes in abundance over the different developmental stages. The authors generalize that there is limited correlation between transcript and protein levels across daily and developmental time. However, since the nature of their proteomic data restricts their analysis to the most abundant proteins in their samples, the general applicability of this conclusion may be quite limited. A more careful analysis of their proteomic data, with a frank discussion of its limitations, is needed. In addition, more details regarding some of their analysis methods are called for.

Specific points:
1) The iTRAQ analysis should be validated with more information on quality control procedures. The correlations among biological replicates, counts of the significant peptides identified, intra-protein peptide mean and standard deviations, coverage of the proteins, and relative abundance in all the proteins identified should be reported.

2) In the classification of proteins and transcripts using a decision tree (Figure 5), it is not indicated how molecules that did not show consistent differences between the EON and EON samples were dealt with. In other words, did each gene or protein have to have higher levels at the EON at each developmental stage for it to be classified as EN? If not, what criteria were used?

3) The authors only compared abundance of proteins that were identified in all of their 8 samples, restricting their analysis to 1673 of the originally identified 2081 proteins in their optimally watered samples. This would further limit their analysis to the most abundant plant proteins and might lead to the exclusion of proteins with genuinely variable levels across their samples. This point should be discussed.

4) The authors should provide a list of all proteins identified in their analysis, not just the ones they identified as variable across their samples.

5) The discussion of the identified GO categories in the results section could be condensed.

6) There is a typo in the supplemental figure 2 legend (reads "< 1.5"; should read "> 1.5").

Reviewer #3 (Remarks to the Author):

The paper by Baerenfaller et al studied four successive developmental stages of Arabidopsis leaf number 6 in two different water regimes by using transcriptome and proteome profiles. For the each developmental stage, they collected samples at the end of the night (EON) and at the end of the day (EOD) to study the growth profiles. They also conducted comparative studies of plants grown under a mild water deficit (SWD) and plants grown under optimal watering conditions (SOW). The authors identified that reduced soil water content (SWD) mainly resulted in reduced final leaf area and reduced final leaf thickness. They also reported reduction in DNA ploidy in SWD leaves. The soil water content did not significantly influence the distinction between developmental stages. The authors observed that the distribution of transcriptome and proteome was similar between SOW and SWD samples.
Overall, the authors have investigated several aspects of systems behavior of Arabidopsis leaf number 6 development at four different growth stages in two different water regimes like reduced soil water content and optimal water content.

The main concern in this study: transcripts showed strong stage and condition dependent diurnal fluctuations but these are not matched with protein level fluctuations. The authors did not try to further confirm these results by using quantitative real time PCR and Western blot analysis. Strong oscillations between EOD and EON were detected for 50.3% of transcripts in SOW and 43.1% in SWD. The authors identified higher accumulation of important clock component genes LHY (AT1G01060) & CCA1 (AT2G46830) at EON. Other genes, TOC1 (AT5G61380) and GIGANTEA (AT1G22770) were higher at EOD. Two defense protein genes PHT4;2 (AT2G29650) and ACD6 (AT4G14400) accumulated to higher levels at EOD. In order to confirm these results, it is better to quantify these genes expression levels by using quantitative real time PCR and Western blots.

The authors should include a list of important up regulated and down regulated genes and proteins along with names, protein molecular weights and protein mass scores in a well prepared tabular format.
The authors should carry out pathway analysis to better understand the pathways of Arabidopsis leaf development.

Page 16 Paragraph 3, line 9: Check the word "BUD".

Figure 5: Clustering of transcript and protein profiles: This figure is prepared in a very complicated way with many abbreviations, it is very difficult to understand in quick glance.

1st Revision - authors' response 19 June 2012

Response to reviewers

Reviewer #1 (Remarks to the Author):

Baerenfaller et al. report the first systems-level analysis of leaf growth, transcript accumulation and protein abundance study for leaf 6 of Arabidopsis thaliana. Strategic consideration of plasticity associated with the developmental by environmental interaction was incorporated into the experimental design by including a comparative examination of transcript and protein accumulation at four carefully defined developmental stages, two points in the diurnal cycle, and two growth conditions. Despite the enormous complexity of the dataset, the presentation is highly engaging. Not too surprisingly, the authors have provided support for a number of earlier observations as well as many first findings. This report and its accompanying dataset will be of considerable interest to plant biologists.

➔ We thank reviewer #1 for this positive statement. We are convinced that our general findings on the different regulatory principles that govern protein and transcript level changes will be of significant interest to the broader science community as well.

Small details:
1) Page 5, second paragraph, last sentence needs addition of the word, 'for' in "could not compensate 'for'..."

➔ This has been added.

2) Figure 5: This is a very important summuative figure, but unfortunately it is extremely difficult to decode. First, the size of the figure needs to be large enough for the reader to see the distinction in color and the labeling. The legend does not provide sufficient support. The authors have presented their code for differential mRNA and protein accumulation in the main text; some of this information ought to be repeated in the legend. The legend should include definitions for abbreviations. Perhaps colored font can be used to help aid interpretation, such as a different colored font can be added. Can information for the number of genes in each branch be included? The red and blue color that is supposed to be evident does not come across.

➔ We thank the reviewer for these recommendations. We have now split Figure 5 into two Figures (new Figures 5 and 6). In the new Figure 5 the decision tree has been completely re-structured and colour coded, and only a sub-tree is displayed (the full decision tree is provided in Supplementary Figure 3). The legend to Figure 5 now briefly summarises the procedure and defines all variables (page 31). In the new Figure 6 the individual panels and the lines indicating stage and EN/ED differences have been enlarged to bring across the red and blue colours. The summary of the number of genes in each panel is now provided in new Supplementary Table 3 because we were concerned that including these numbers for proteins and transcripts in both experiments would make the figure more confusing again. In addition to the example patterns displayed in Figure 6 we now provide all the patterns for proteins and transcripts in SOW and SWD in Supplementary Figures 4-7. We expect that these changes will make the information now more easily accessible to the reader.

Reviewer #2 (Remarks to the Author):

Baerenfaller and colleagues present a comprehensive analysis of leaf characteristics across developmental time in plants grown under optimal and mild water stress conditions. They carefully analyzed growth parameters of the first adult rosette leaf (leaf #6) to define four distinct growth stages. They found that the water limiting conditions slowed the pace of growth and resulted in smaller plants with fewer cells and reduced polyploidy. For genomic analyses, they defined four developmental stages and collected samples of plants grown in both water conditions at the end of the night (EON) and end of the day (EOD). Importantly, the same biological samples were used for transcriptomic (using a tiling array) and proteomic analysis (using iTRAQ). The authors clustered the protein and transcript data to find distinct patterns in accumulation and compare these between the two growth conditions.

In their analysis of transcript levels, the authors report large differences across the developmental and time-of-day samples. They also find that the water-limited plants show reduced amplitude of oscillations in gene expression compared to the plants grown in optimal water conditions.

In contrast, they find that very few proteins show differences in abundance across their time-of-day samples. The iTRAQ technique has limited sensitivity, allowing only a small fraction of the proteome to be quantifiably assessed in these experiments (2081 and 1509 proteins in the water optimal and water limiting conditions, respectively). In addition, iTRAQ has previously been reported to underestimate actual changes in protein levels (Pierce et al, Molecular and Cellular Proteomics, 2008). Of the proteins that could be quantified, almost none showed daily changes in abundance and only ~25% showed changes in abundance over the different developmental stages.

➔ For the analysis of the iTRAQ proteomics data we had already carefully considered the issues raised by the reviewer concerning accuracy and precision in iTRAQ quantitation. In a study published by Karp and colleagues (MCP, 2010) the authors showed that precision of the data is affected by variance heterogeneity and that accuracy is compromised as ratios are compressed towards 1, leading to underestimation of the ratios. They recommended using a variance-stabilising normalisation to address the error structure. In our analysis we only included peptide spectrum assignments that had an expect value < 0.05, an ion score > 24, and for which all seven ratios had a positive value, which excluded a considerable amount of low signal data. As shown in the ratio/intensity plot in Supplementary Figure 2, this solved the issue of high variability in the low signal range. We did not normalise the data to correct for the underestimation of ratios, because we processed the protein and transcript data in the same way. In addition, we do not expect that underestimation of high ratios will skew our analyses, because this mainly affects proteins with high relative protein abundance differences. Since we could use a relatively low 1.5-fold threshold by combining the fold-change cut-off with rigorous statistical significance of the change, we have full confidence in the sensitivity of iTRAQ and the validity of our data as well as their significance. Further, as is evident from the iTRAQ data, we reproducibly detect changes up to nearly 12-fold (e.g., AT2G37760, AKR4C8, pattern U-E-E-E), which demonstrates that also large abundance changes could be detected using iTRAQ. In addition, finding significant changes in 27% of all identified proteins despite of rigorous statistical testing (including correction for multiple testing) is far more than what is usually observed in quantitative proteomics data. Changes can typically be found in only 5-10% of the

identified proteins, even if less strict tests are used. Our data represent a considerable change in the leaf proteomes across the four growth stages, further confirming that we can reliably detect protein abundance changes.
We have revised the discussion to clarify and strengthen this point. We have also explained that our rigorous statistical testing (including the correction for multiple testing) and the applied cut-offs may have produced false negatives, but this makes the proteins that do change in our dataset even more significant (page 17, last paragraph and page 18, first paragraph; Supplementary Information, page 4, first paragraph).

The authors generalize that there is limited correlation between transcript and protein levels across daily and developmental time.

➔ We found that the proteins and transcript correlate quite well in their general growth patterns with some notable exceptions, but not between EOD and EON. This was already discussed in the manuscript, but we attempted to make this clearer in the revised version. As mentioned above, the positive correlation across developmental changes confirms that we can reliably detect changes in protein levels.

However, since the nature of their proteomic data restricts their analysis to the most abundant proteins in their samples, the general applicability of this conclusion may be quite limited. A more careful analysis of their proteomic data, with a frank discussion of its limitations, is needed.

➔ We have revised the discussion to address the concern of the reviewer (page 17, last paragraph and page 18, first paragraph).

In addition, more details regarding some of their analysis methods are called for.

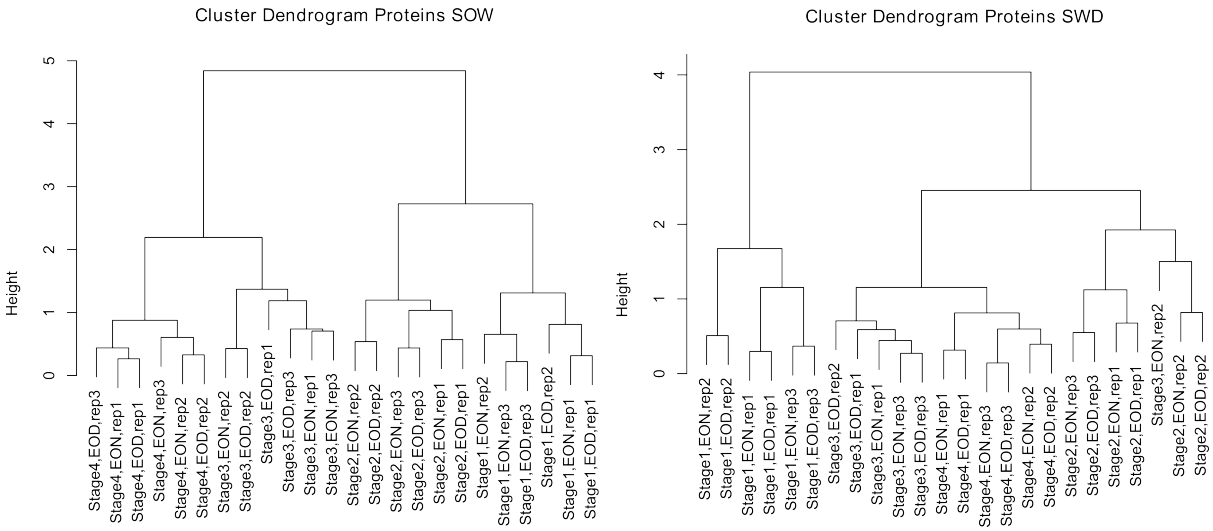
➔ We thank the reviewer for detailing the additional supporting evidence that should be made more easily accessible to the reader. As detailed below, we have now included much of the information that is available in the AGRON-OMICS Leaf Database (LDB) and/or in pep2pro into the Supplementary Material and provided additional information.

Specific points:
1) The iTRAQ analysis should be validated with more information on quality control procedures. The correlations among biological replicates, counts of the significant peptides identified, intra-protein peptide mean and standard deviations, coverage of the proteins, and relative abundance in all the proteins identified should be reported.

➔ As quality control and to assess the source of variation in the data we had performed PCA analyses, which demonstrated that the difference between the different growth stages is the main contribution to the variability and the high reproducibility between biological replicates (Figure 4). We have now also included a correlation analysis in the Supplementary Material (Supplementary Table 28). The Figure below shows the hierarchical clustering of the correlation data and demonstrates that the result from the clustering corresponds to what we have observed in the PCA analysis. Together, the proteomics data are clearly separated for the different growth stages, but not for the differences between EOD and EON.

➔ For the quantification of the proteins we had averaged the quantitative information in all the spectra for the individual proteins, which is equal to calculating a weighted average over the peptides according to the number of spectra annotated to each peptide. We preferred this approach because of the experimental set-up that we adopted with 4 (partly overlapping) technical replicates for each biological replicate (Supplementary Tables 24, 25). This makes the quantification based on peptide means very complicated, especially with regard to error propagation (how to combine the standard deviation of the intra-protein peptide means with that of the intra-peptide spectrum means?). The lists of all identified proteins, the number of contributing spectra, and the standard deviation are now provided in Supplementary Tables 26 (SOW) and 27 (SWD). Information on the contributing peptides and the number of spectra per peptide can be accessed in the pep2pro database, in which we will make all information on the protein and peptide identifications and the individual spectra publicly available upon acceptance of the manuscript. The information

on protein coverage cannot simply be added to the protein identification tables because it is specific for splice variants that we did not distinguish in our analysis. However, we provide the protein coverage for each splice variant in the pep2pro database.



2) In the classification of proteins and transcripts using a decision tree (Figure 5), it is not indicated how molecules that did not show consistent differences between the EON and EON samples were dealt with. In other words, did each gene or protein have to have higher levels at the EON at each developmental stage for it to be classified as EN? If not, what criteria were used?

➔ We re-structured Figure 5 and revised the figure legend (page 31) to clarify that data, which do not meet the criteria for significant changes between end-of-day and end-of-night, are classified as 'E'. As explained in Materials and Methods, the p-values for diurnal changes resulted from the ANOVA analysis, in which stages and day-time were treated as separate effects. Therefore it was not required that abundance levels between EOD and EON had to be significantly different at each developmental stage, but only that there was an overall significant difference.

3) The authors only compared abundance of proteins that were identified in all of their 8 samples, restricting their analysis to 1673 of the originally identified 2081 proteins in their optimally watered samples. This would further limit their analysis to the most abundant plant proteins and might lead to the exclusion of proteins with genuinely variable levels across their samples. This point should be discussed.

➔ We did the statistical tests and calculated the fold change for all 2081 proteins. The results from the statistical analyses and the fold changes are provided for all proteins detected in both experiments in the AGRON-OMICS LDB, but we have now also included the information for all transcripts and proteins in SOW and SWD in Supplementary Table 29. Proteins that did not have a value for each sample were excluded only for the decision tree because the pair-wise comparisons were an absolute prerequisite for the classification. As the result, only 30 proteins in SOW and 16 in SWD meeting the fold-change and significance thresholds were not included in the decision tree. The missing values are from all stages and only few of the remaining pairwise comparisons had p-values < 0.05. Also, none of these proteins showed a significant diurnal change. We have now included these proteins in the Supplementary Material (Supplementary Table 30) to demonstrate that the data are similar but have gaps. These gaps cannot be filled when working with relative data, because as in the data reported here placing a 1 (or 0 in log-scale) for the sample/reference ratio cannot be justified. Also imputing is not possible, because there are no data available for a given stage based on which the values could be estimated. Together, this shows that we did not exclude an important fraction of proteins with genuinely variable levels and that our filter did not compromise the results of our analyses.

4) The authors should provide a list of all proteins identified in their analysis, not just the ones they identified as variable across their samples.

➔ This information is provided in the LDB, but we have now also added it to the Supplementary Material (Supplementary Tables 26 and 27)

5) The discussion of the identified GO categories in the results section could be condensed.

➔ The discussion of the identified GO categories was already significantly condensed after several rounds of revisions by the co-authors. We feel that the current length is required to provide the reader with a meaningful discussion of key GO category examples that illustrate the novelty and significance of our dataset.

6) There is a typo in the supplemental figure 2 legend (reads "< 1.5"; should read "> 1.5").

➔ This was corrected.

Reviewer #3 (Remarks to the Author):

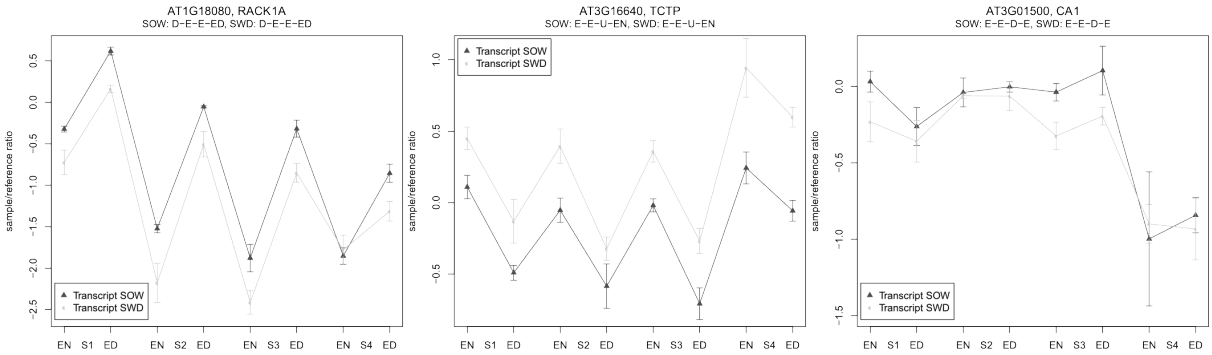
The paper by Baerenfaller et al studied four successive developmental stages of Arabidopsis leaf number 6 in two different water regimes by using transcriptome and proteome profiles. For the each developmental stage, they collected samples at the end of the night (EON) and at the end of the day (EOD) to study the growth profiles. They also conducted comparative studies of plants grown under a mild water deficit (SWD) and plants grown under optimal watering conditions (SOW). The authors identified that reduced soil water content (SWD) mainly resulted in reduced final leaf area and reduced final leaf thickness. They also reported reduction in DNA ploidy in SWD leaves. The soil water content did not significantly influence the distinction between developmental stages. The authors observed that the distribution of transcriptome and proteome was similar between SOW and SWD samples.
Overall, the authors have investigated several aspects of systems behavior of Arabidopsis leaf number 6 development at four different growth stages in two different water regimes like reduced soil water content and optimal water content.
The main concern in this study: transcripts showed strong stage and condition dependent diurnal fluctuations but these are not matched with protein level fluctuations. The authors did not try to further confirm these results by using quantitative real time PCR and Western blot analysis.

➔ On request of the reviewer we performed quantitative RT-PCR and qualitative Western blot analyses for a small subset of genes. Unfortunately there are only few commercially available plant antibodies and the majority of them are polyclonal antibodies with limited specificity and sensitivity. For the validation we used tested antibodies for DPE2, GSTF2 and POR to confirm interesting variation patterns of selected proteins. In parallel we performed qRT-PCR analyses for their mRNAs to confirm their transcript variation patterns in SWD (we were limited here to the SWD experiment as the remaining RNA from the SOW experiment was used for the qRT-PCR plastid transcript data). As shown in new Supplementary Figure 13, the variation patterns between EOD and EON were largely consistent in the tiling array and qRT-PCR data. The mRNA variation over the developmental stages was identical for PORC and generally corresponded for DPE2, PORB and GSTF2 in both datasets. The signals for POR, GSTF2 and DPE2 in the Western blots shown in new Supplementary Figure 14 also agreed with the iTRAQ data. Based on the signals in the Western blots, no reliable statements concerning diurnal protein level changes can be made.

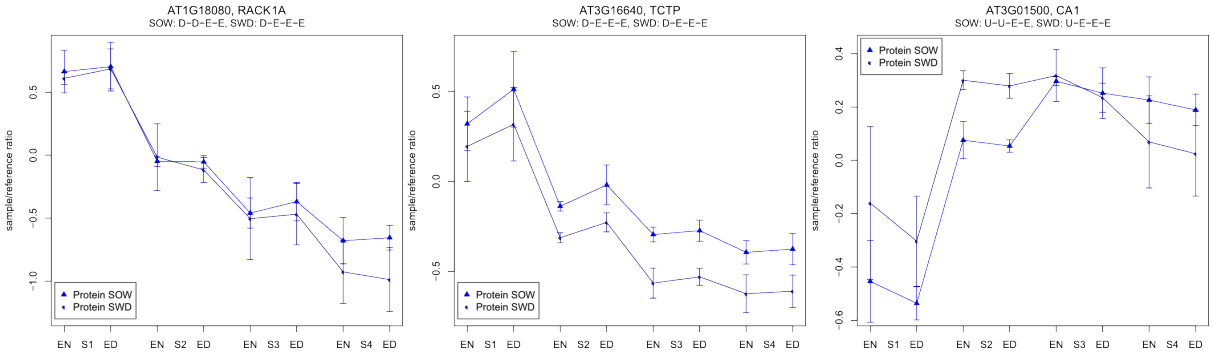
Although we found a good agreement between the quantitative data reported in our manuscript with the results from the alternative analysis methods, we are well aware of the fact that conclusions from such a small subset of genes are limited. As an additional approach for the validation of our data we would therefore like to point out the good correlation we have observed for the protein and transcript data in SOW and SWD (mean correlation transcripts = 0.68; mean correlation proteins = 0.51). Some examples are shown in Supplementary Figures 13 and 14 and in the Figure below (the plots with combined

patterns for all proteins and transcripts are provided in the LDB). Considering that SOW and SWD data were obtained from two subsequent and independent experiments with independent tiling array hybridisations and mass spectrometry measurements performed over a time period of more than 1.5 years we think this correlation is remarkable and further confirms the high quality of the provided data.

AGRONOMICS1 tiling array



iTRAQ



Strong oscillations between EOD and EON were detected for 50.3% of transcripts in SOW and 43.1% in SWD. The authors identified higher accumulation of important clock component genes LHY (AT1G01060) & CCA1 (AT2G46830) at EON. Other genes, TOC1 (AT5G61380) and GIGANTEA (AT1G22770) were higher at EOD. Two defense protein genes PHT4;2 (AT2G29650) and ACD6 (AT4G14400) accumulated to higher levels at EOD. In order to confirm these results, it is better to quantify these genes expression levels by using quantitative real time PCR and Western blots.

➔ We pointed out the clock genes in particular because in our data their transcripts levels do exactly what they are supposed to do. This, together with published evidence, supports the reliability, reproducibility and validity of our data. There is currently no possibility to track the protein levels for these genes because antibodies are not available. The transcripts level changes for the other two genes were shown not because these are particularly interesting candidates, but to exemplify that the diurnal transcript level fluctuations are sometimes stage specific. This is an important finding that we discovered by a statistical analysis of the full dataset and this is discussed in the manuscript.

The authors should include a list of important up regulated and down regulated genes and proteins along with names, protein molecular weights and protein mass scores in a well prepared tabular format.

➔ The information on regulated proteins and genes with gene annotation as well as the clustering information was already provided in the Supplementary Tables of the original submission (now Supplementary Tables 4-7 in Dataset 1). We did not provide protein molecular weights because these are specific for splice variants that we did not distinguish in our analysis. However, we provide the molecular weight of each splice variant together with all protein information in the pep2pro database. We apologise, but we do not understand what the reviewer means by “protein mass score”.

The authors should carry out pathway analysis to better understand the pathways of Arabidopsis leaf development.

➔ For the analysis of the high-throughput data we integrated the molecular and phenotypic profiles of leaf growth. As described in the manuscript this included the search for molecular profiles that reflect the functional states of the leaf and we spent considerable effort identifying the processes that act during leaf development. We do not understand what the reviewer means by 'pathways of Arabidopsis leaf development', as we are not aware of such information that could have greatly helped the interpretation of our data. Instead, our data break new ground and provide significant new information on molecular processes that will greatly help to understand leaf development and physiological changes at each developmental stage.

Page 16 Paragraph 3, line 9: Check the word "BUD".

➔ This was corrected.

Figure 5: Clustering of transcript and protein profiles: This figure is prepared in a very complicated way with many abbreviations, it is very difficult to understand in quick glance.

➔ Figure 5 has been re-structured and the figure legend revised to make it more easily accessible to the reader (also see comment of reviewer 1).

2nd Editorial Decision 12 July 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the two referees who agree to evaluate your revised study. As you will see, the referees felt that the revisions made had improved this work, and Reviewer #3 is now supportive. Reviewer #2, however, feels that some of the claims made in this work need to better qualified, and we have some final minor requests, which we would ask you to carefully address in a revision of the present work.

The Reviewer #2 clearly felt that the claims made in this manuscript, particularly regarding the lack of circadian oscillations in protein levels, need to be stated with more caution. This reviewer felt that is important to clearly recognize the high variation in the proteomic measurements and the lack of sensitivity which prevents detection of many low-medium abundance proteins. Statements regarding the generality of this observed lack of oscillations need to be altered to make clear that the data at present can only provide reliable information about relatively abundant proteins.

In addition, we ask you to address the following format and content issues:

1. Please provide reviewer login links for the transcriptomic and proteomic datasets deposited at PRIDE and ArrayExpress so that we can verify these submissions.

2. In addition to our capacity to host datasets in our supplementary information section, we provide a new functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). We encourage you to supply figure source data for the experimental results not covered by the transcriptomic and proteomic datasets (e.g. Fig. 1). Please see our Instructions of Authors for more details on preparation and formatting of figure source data (<http://www.nature.com/msb/authors/index.html#a3.4.3>). I recognize that some of these data are already covered to some degree in the Supp. Tables, but, in general, they may be more accessible to readers as figure source data, and we encourage authors to supply replicate measurements whenever possible.

3. Figure Quality

- Some of the last reviewer's concerns about figure quality seem to arise from conversion problems within our submission system. Certainly the figures in the Supplementary Information pdf look much better in the Word Doc than in the pdf created by our system. To avoid this, you may wish to

directly submit a pdf file for the Supplementary Information document.

- For the main manuscript figures, please make sure all text is readable when the figure is formatted to typical single page dimensiosns (should remain >9pt), and make sure that all text and line-art remains clear and crisp even when zooming in. You may obtain the best results and the smallest file sizes if the figures are saved directly in EPS or PDF formats from a high-quality vector graphics program like Illustrator or Inkscape.

- Please provide the main manuscript figures only as individual image files, and remove them from the main manuscript Word document.

4. Please provide three to four 'bullet points' highlighting the main findings of your study.

5. Please provide a 'standfirst text' summarizing your study in one or two sentences (approx. 250 characters).

6. Please provide a "thumbnail image" (width=211 x height=157 pixels, jpeg format), which can be used to highlight your paper on our homepage.

Please resubmit your revised manuscript online, with a covering letter listing amendments and responses to each point raised by the referees. Please resubmit the paper **within one month** and ideally as soon as possible. If we do not receive the revised manuscript within this time period, the file might be closed and any subsequent resubmission would be treated as a new manuscript. Please use the Manuscript Number (above) in all correspondence. Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Referee reports:

Reviewer #2 (Remarks to the Author):

The revised manuscript "Systems-based analysis of Arabidopsis leaf growth reveals adaptation to water deficit" provides a careful phenotypic analysis of leaf growth under two different environmental conditions and attempts to determine mechanisms and pathways underlying these differences by transcriptomic and proteomic characterization.

Although the authors appear to have carried out their experiments with care, the current state of the field of quantitative proteomics necessarily limits their ability to generate reproducible and quantitative data. This is illustrated by the often-low correlations (Pearson's r ranging from 0.31 to 0.81; mean r =0.58) between their biological replicates in the proteomic analysis (Supplemental Table 28). Given such variability between samples, one should interpret the results of these experiments with care, especially when making quantitative arguments.

My main concern is that they attempt to generalize quantitative observations (with limited correlation between replicates) made for a small fraction of cellular proteins (in the best case about 2000 proteins, or ~12% of expressed genes) to the entire proteome. Their analysis is thus necessarily limited to the most abundant proteins in the cell; this is a very skewed sample from a biological standpoint. In particular, statements such as "why over half of the leaf transcriptome shows diurnal fluctuations throughout growth if this has little impact on protein abundance" and "Thus, our discovery (lack of diurnal protein oscillations compared to transcript oscillations) that this is a general rule" seem misleading. The authors likely recognize the fact that they have assayed a biologically skewed set of proteins, as they also state "For example, for enzymes of central metabolism the relative amount of transcript to protein is so low that in a leaf it may take several days for a change in transcript level to cause a major change in protein level." The authors should

not attempt to draw sweeping conclusions about the entire genome based upon their analysis of a subset of proteins that may be highly atypical.

Reviewer #3 (Remarks to the Author):

This is a much improved version of the manuscript. The authors changed figure 5 and made it into two new figures (Figure 5 and figure6). They are easier for reading. Authors also conducted real time PCR and western blot analyses to validate the results as suggested. The results are consistent with the transcript changes. The authors included supplementary figure 13 and 14 to present real time PCR and western blot results. However, the quality of these pictures is poor. The Labels in these figures are not clear enough to read. IN addition, the labels of figure 4 and 3 in the main text are also not clear enough to read.

Overall, authors made major changes according to the suggestions of the reviewers. I think this version of manuscript can be considered for acceptance by Molecular Systems Biology with a few minor revisions.

2nd Revision - authors' response 17 July 2012

Response to reviewers

Reviewer #2 (Remarks to the Author):

The revised manuscript "Systems-based analysis of Arabidopsis leaf growth reveals adaptation to water deficit" provides a careful phenotypic analysis of leaf growth under two different environmental conditions and attempts to determine mechanisms and pathways underlying these differences by transcriptomic and proteomic characterization.

Although the authors appear to have carried out their experiments with care, the current state of the field of quantitative proteomics necessarily limits their ability to generate reproducible and quantitative data. This is illustrated by the often-low correlations (Pearson's r ranging from 0.31 to 0.81; mean r =0.58) between their biological replicates in the proteomic analysis (Supplemental Table 28). Given such variability between samples, one should interpret the results of these experiments with care, especially when making quantitative arguments.

My main concern is that they attempt to generalize quantitative observations (with limited correlation between replicates) made for a small fraction of cellular proteins (in the best case about 2000 proteins, or ~12% of expressed genes) to the entire proteome. Their analysis is thus necessarily limited to the most abundant proteins in the cell; this is a very skewed sample from a biological standpoint. In particular, statements such as "why over half of the leaf transcriptome shows diurnal fluctuations throughout growth if this has little impact on protein abundance" and "Thus, our discovery (lack of diurnal protein oscillations compared to transcript oscillations) that this is a general rule" seem misleading. The authors likely recognize the fact that they have assayed a biologically skewed set of proteins, as they also state "For example, for enzymes of central metabolism the relative amount of transcript to protein is so low that in a leaf it may take several days for a change in transcript level to cause a major change in protein level." The authors should not attempt to draw sweeping conclusions about the entire genome based upon their analysis of a subset of proteins that may be highly atypical.

➔ We recognize that our finding applies to the subset of proteins that we could measure in our proteome analysis. We have therefore carefully reworded the relevant sections of the manuscript related to the limited diurnal fluctuations of the protein levels (page 6, line6, 'Leaf transcriptome and proteome dynamics..' substituted with 'Dynamics of leaf transcriptome and the measured proteome…'; page 6, line 28, 'at the proteome level' substituted with 'at the level of the measured proteome'; page 9, last line, '…this is a general rule affecting over 1700 quantified proteins…' substituted with '… this affects over 1700 quantified proteins'; page 10, line 1, introduction of the sentence 'However, we cannot exclude that our finding applies mainly to high-abundance proteins that can be measured in a large-scale proteome analysis and that low-abundance proteins may, at least in part, fluctuate between EOD and EON in correspondence with their transcripts.'; page 18, line 4, addition of '…in the detected proteome…').
We would like to emphasise, however, that even if the detected subset of proteins might not be representative for the whole proteome, we do see diurnal level fluctuations for 74% of the subset of transcripts for which we also have quantitative proteome data. Therefore, our results are novel and intriguing, and the question remains why transcripts for over 1700 quantified proteins show diurnal fluctuations if proteins do not fluctuate correspondingly.
We have modified our conclusion in the discussion concerning the lack of diurnal protein fluctuations to clarify that we are referring here to a sub-sample of the whole genome (page 18, line 20, 'why over half of the leaf transcriptome shows … ' substituted with '…why 74% of the transcripts in the transcriptome subset for which we have proteome data shows…')

