Rapid and Dynamic Alternative Splicing Impacts the Arabidopsis Cold Response Transcriptome

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Review timeline:

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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2018-00177-RA 1st Editorial decision – revision requested April 9, 2018

We have received reviews of your manuscript entitled "Rapid and dynamic alternative splicing impacts the Arabidopsis cold response transcriptome." On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in The Plant Cell. This acceptance is contingent on revision based on the thoughtful comments of our reviewers.

Please highlight all changes and include a detailed annotation to changes to the text, with line numbers, and noting your responses to the comments.

Reviewer comments and author responses:

Reviewer #1:

Alternative splicing (AS) plays an important role in expanding the information encoded by eukaryotic genomes. It largely increases proteome and transcriptome diversity. Recent findings strongly indicate that intensive AS occurs in plant systems. AS probably plays an important role under changing environments. This manuscript reported a large-scale, time-series RNA-seq to examine the dynamics of differential AS and differential gene expression in response to low temperature. With a newly developed analysis pipeline, the authors determined differential expression (DE), differential alternative splicing (DAS) and differential transcript usage (DTU). They discovered the total of 8,949 Arabidopsis genes showing DE or DAS. By comparing with previous data, the authors identified novel cold response genes showing regulation at transcription and/or splicing. They validated one of the newly found DAS gene encoding the splicing factor U2B"-LIKE and confirmed its function in cold acclimation. A final model was proposed.

The manuscript delivers a complete survey for transcriptome when Arabidopsis expose to cold. It is well written and organized. The data and description in this manuscript are reasonably sound. As suggested, large-scale biology articles published in The Plant Cell should fall in these criteria: of exceptional quality and potentially high impact, providing novel insight into plant biology, presenting significant new methodology or computational/analytical tools, or introducing a new resource of significant value to the broader plant science community. I find this manuscript fits well into these criteria. Although similar studies have been done previously, this report makes important contributions by
providing new and complete information to reveal transcriptome patterns under cold stress in a high resolution. I expect this paper will be of great interest to researchers focusing in cold responses as well as pre-mRNA splicing. Detailed comments are shown as below. Significance: The first complete study with new analyses to show transcriptome changes under low temperature.

Point 1. Weakness: Global analysis of cold-induced transcription and AS in Arabidopsis has been reported.

RESPONSE: We do not see this as a weakness. There are many global analyses of gene expression and some of AS in response to a range of stresses in Arabidopsis and other plants. We did not point out explicitly the limitations (and the poor understanding of the limitations) of the most widely used RNA-seq analysis programs which mean that the majority of published RNA-seq papers, especially AS papers, would benefit from re-analysis using the up-to-date tools now available. We certainly hope that our paper will demonstrate what is possible for RNA-seq analyses and that researchers (and editors) will begin to include high quality AS analysis routinely in RNA-seq analyses.

Point 2. The authors generated very useful data sets, however it is too bad that they did not analyze further to dig out more information. For example, functional enrichment for up- and down-regulated DE and DAS genes. Because the data is from a time series experiments, time-ordered, cold-dependent gene co-expression networks can also be done to reveal transcription network (or splicing network) of cold response in plants.

RESPONSE: The amount of primary information in this work is huge and therefore we have performed analyses to illustrate the main findings. We address the specific comments here:

a) the up- and down-regulated genes are shown for each contrast group (e.g. time-point comparison) in Suppl Fig 3. However, some genes/transcripts are up- and down-regulated in different contrast groups and therefore we performed the enrichment analyses on clusters of gene and transcript expression profiles (Suppl. Fig 5 and 7; Figure 4 and 5). This is much more refined than looking at up- and down-regulated genes in each contrast group separately.

b) The time-series data can, of course, be used to construct gene and splicing networks. However, this is not trivial given the size of the datasets and we have begun to explore the best way to address this.

Point 3. The link between U2B⁺-LIKE and cold response is slightly weak. Although the authors tested splicing patterns for some genes, not many of them can be associated with cold response.

RESPONSE: The selection of the genes/AS events for use in RT-PCR was based on our analysis which identified DAS genes with rapid and large changes in AS in response to cold. They are therefore associated with the cold response but many have not been identified in previous studies as cold-responsive. See more detailed note above (2nd last paragraph on p1).

Point 4. The results do not fully support the model being made especially on splicing. As the authors hypothesize that calcium signaling and MAPK cascade initiate downstream gene regulation, can the authors provide evidence based on RNA-seq data?

RESPONSE: We see changes in expression and/or AS of MAP kinases, calcium signalling components, calcium-dependent kinases etc. It is not possible from the RNA-seq data to directly link phosphorylation activity to changes in AS but it is therefore a reasonable expectation and model. The model is based on the well-documented calcium and MAPK signalling pathways in the cold; the identification of phosphorylation of 22 RNA-interacting proteins (de la Fuente et al., 2006) of which 16 are DAS genes and 7 are in the early AS gene set; and extensive work in animal systems on phosphorylation of splicing factors (also by calcium signalling) to affect activity and localisation. We feel there is enough data already to suggest involvement of Ca and MAPKs and expect that rapid changes in AS are brought about by rapid phosphorylation to activate/deactivate splicing factors. Readers will appreciate that this is just a hypothesis. Of course, we are testing it and it will be a subject of a separate paper.

Reviewer #2:

In this large scale biology manuscript, Calixto and co-workers use cold-stress as a paradigm to monitor changes in differential gene expression and alternative splicing in plant response to environmental stress, during the recovery phase, and during long term stress.
For this, Arabidopsis thaliana plants were sampled throughout one day at ambient temperature (20°C), on the first day after transfer to low temperature (4°C), and on the fourth day at 4°C. Importantly, all samples were paralleled by samples harvested at the very same time points at 20°C. This rules out effects causes by changes in circadian clock progression at low temperatures.

The samples were processed for paired-end sequencing. A total of 8,949 significant changes were identified, with 1,647 only at the level of alternative splicing and 795 both at level of differential expression and alternative splicing.

Crucial to the success of the analysis was the use of the new reference transcriptome ATRTD2-QUASI previously developed by the authors.

A major outcome of the manuscript is the identification of cascades of alternative splicing events on top of changes in transcript levels that shape the cold-responsive transcriptome, ultimately ensuring adaptation and survival at low temperatures. The data also led to the identification of a novel splicing regulator with a function in cold tolerance.

Overall, this is an excellent example of a well-conceived "large scale biology" experiment resulting in high quality data that will be met with great interest in the community. The manuscript is very well written. I have only a few points to consider.

Point 1. In contrast to most cold time courses done before, the authors start the cold treatment at dusk. They observe a different timing of induction of CBF, a key regulator of the cold transcriptome, compared to previous experiments. Moreover, 1708 novel DEGs are identified in this study. While some of this may be due to the improved reference transcriptome, it may also relate to the different timing of stress application.

RESPONSE: We discuss the various possible reasons for our detection of more novel DE genes and non-detection of some previously identified genes in the Results. The reviewer also noted the difference in the timing of induction of the CBFs that we see in this analysis. We have provided an explanation for this – although the first time-point of significant DE for the three CBF genes was T11 (6h after cold application), all three genes showed significant fold-changes at T10 (the first time-point of cold) but the p-values did not meet the significance criteria.

Point 2. Would it be possible to conduct a systematic / more comprehensive comparison of expression profiles of key cold response transcripts by a more extensive comparison of data gathered when temperature is lowered in the morning vs. the novel study of the authors with temperature lowered in the evening?

This would also allow conclusions about gating of the cold response by the circadian clock.

RESPONSE: We do not think that it is possible to make more detailed and systematic comparisons with other data to be able to identify the effects of lowering temperature at dawn versus dusk. It would be interesting to re-address gating with our methods but this would require a specific experiment.

In general, such comparisons are difficult to conduct at a detailed level. The comparison of DE genes identified here with those identified by microarray and gene-level RNA-seq (Figure 2) is the most meaningful comparison that can be made because of the different experimental systems (cold shock/temperature reduction; day length; sampling times; age of plants; ecotypes – see Suppl dataset 3), amount and accuracy of data, RNA-seq analysis programs and methods, and significance criteria.

Point 3. DTU is not so widely used in the plant field as yet. DTUs arise by alternative splicing, alternative transcription start sites, alternative polyadenylation or combinations thereof. It seems that the authors look at DTUs only with respect to alternative splicing. Can the data be used to gain some insights in changes in alternative transcription start sites or alternative polyadenylation?

RESPONSE: As our focus is on alternative splicing, we have not addressed alternative transcription start sites (TSS) or alternative polyadenylation (pA). At present, we do not think that it is possible to analyse effectively changes in TSS and pA. This would require, firstly, the systematic definition of transcripts with bona fide alternative TSS and pA sites and, secondly, the ability to quantify accurately such transcripts. Single molecule sequencing is likely to help to define such transcripts but their quantification in RNA-seq data is likely to require some development - when constructing AtRTD2, we noticed that transcripts from the same gene with different 5’ and 3’ lengths could show drastic differences in accuracy of quantification (Zhang et al., 2017). We overcame this for AS by developing AtRTD2-QUASI.
Point 4. The authors find rapid alternative splicing of SUF4, an activator of FLC expression. Is this thought to have any relevance for FLC expression, as FLC shows a slow, gradual reduction upon prolonged cold exposure?

RESPONSE: We do not know the functions of the different SUF4 isoforms and their effects on FLC. In addition to SUF4, other FLC regulators, SDG10 and SDG25 also have altered expression/AS in the cold – however, in the time course of this experiment, the expression of FLC does not change significantly. In addition, autonomous pathway and other flowering time genes (FCA, FY, FLD, FLM, COL1, COL2, MAF2, MAF3...) also have significantly altered expression/AS. It is possible that SUF4 has other functions or that it and the other flowering time genes act to slow flowering as an initial response to lowering temperature but currently we do not know how such changes in expression/AS may affect flowering in prolonged cold.

Point 5. Fig. 3 A cluster of genes gain rhythmic expression in the cold. For comparison, the behavior of selected core clock genes under these conditions should be shown to monitor clock progression.

RESPONSE: We are aware of changes in rhythmicity in a number of genes with some losing rhythmicity (e.g. U2B-like – Figure 8A) or gaining rhythmicity in the cold. These differences may reflect photoperiodic and/or circadian clock regulation. However, at this time we have not conducted experiments to infer that the changes are related to the clock. Therefore, we do not think that a Figure with these profiles adds to this paper; the changes in expression/AS of the core clock genes are available on the dashboard.

Reviewer #3:

In this study, the authors present a comprehensive, high-throughput, time-series RNA-seq dataset describing the changes in Arabidopsis plants during fast cold response as well as cold acclimation and freezing tolerance. While the cold response in plants has been subject of research for decades, previous studies mostly focused on changes in transcription. The dynamics of alternative splicing (AS) in response to cold had not yet been analysed.

The authors employ RNA-seq of numerous samples taken over a well-designed time course and reveal a striking AS response. They identify over 2400 genes undergoing differential AS, in addition to the ~7300 differentially expressed genes. More importantly, over 1600 genes are regulated only at the AS level, the vast majority of which were not previously identified as cold-responsive. Among those, U2B'-like, a splicing factor the authors show to be required for cold acclimation and freezing tolerance. This paper provides tremendously important resource for future research efforts to resolve the inner workings of how plants deal with cold temperatures.

Importantly, the authors have used state-of-the-art methods and applied stringent statistical frameworks. What's more, the development of tools and approaches to analyse differential splicing has played a central role in this study, and these are now available to the scientific community. Previously, the authors assembled and published the Arabidopsis reference transcriptome AtRTD2 and its version AtRTD2-QUASI that has enabled current analyses. Here, they provide the pipeline for analysis of differential gene expression, differential AS and differential transcript usage. Moreover, the interactive viewer of the gene/transcript expression profiles in the time-series data will be of immediate value for plant researchers.

TPC2018-00177-RAR1 2nd Editorial decision – acceptance pending  April 25, 2018

We are pleased to inform you that your paper entitled "Rapid and dynamic alternative splicing impacts the Arabidopsis cold response transcriptome" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor May 5, 2018