

*Rapid report***AtRTD – a comprehensive reference transcript dataset resource for accurate quantification of transcript-specific expression in *Arabidopsis thaliana***

Author for correspondence:

John W. S. Brown

Tel: +44 1382 568777

Email: j.w.s.brown@dundee.ac.uk

Received: 19 March 2015

Accepted: 5 June 2015

Runxuan Zhang^{1*}, Cristiane P. G. Calixto^{2*}, Nikoleta A. Tzioutziou², Allan B. James³, Craig G. Simpson⁴, Wenbin Guo^{1,2}, Yamile Marquez⁵, Maria Kalyna⁶, Rob Patro⁷, Eduardo Eyra^{8,9}, Andrea Barta⁵, Hugh G. Nimmo³ and John W. S. Brown^{2,4}

¹Informatics and Computational Sciences, The James Hutton Institute, Invergowrie, Dundee, DD2 5DA, UK; ²Plant Sciences Division, College of Life Sciences, University of Dundee, Invergowrie, Dundee, DD2 5DA, UK; ³Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, G12 8QQ, UK; ⁴Cell and Molecular Sciences, The James Hutton Institute, Invergowrie, Dundee, DD2 5DA, UK; ⁵Max F. Perutz Laboratories, Medical University of Vienna, Dr Bohrgasse 9/3, 1030 Vienna, Austria; ⁶Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria; ⁷Computer Science Department, 1422 Computer Science, Stony Brook University, Stony Brook, NY 11794-4400, USA; ⁸Computational Genomics, Universitat Pompeu Fabra, 08002 Barcelona, Spain; ⁹Catalan Institution of Research and Advanced Studies (ICREA), 08010 Barcelona, Spain

Summary*New Phytologist* (2015) **208**: 96–101

doi: 10.1111/nph.13545

Key words: alternative splicing, *Arabidopsis thaliana*, high resolution reverse transcription (HR RT)-PCR, RNA-sequencing (RNA-seq), SAILFISH, SALMON, transcripts per million.

- RNA-sequencing (RNA-seq) allows global gene expression analysis at the individual transcript level. Accurate quantification of transcript variants generated by alternative splicing (AS) remains a challenge. We have developed a comprehensive, nonredundant *Arabidopsis* reference transcript dataset (AtRTD) containing over 74 000 transcripts for use with algorithms to quantify AS transcript isoforms in RNA-seq.
- The AtRTD was formed by merging transcripts from TAIR10 and novel transcripts identified in an AS discovery project. We have estimated transcript abundance in RNA-seq data using the transcriptome-based alignment-free programmes SAILFISH and SALMON and have validated quantification of splicing ratios from RNA-seq by high resolution reverse transcription polymerase chain reaction (HR RT-PCR).
- Good correlations between splicing ratios from RNA-seq and HR RT-PCR were obtained demonstrating the accuracy of abundances calculated for individual transcripts in RNA-seq.
- The AtRTD is a resource that will have immediate utility in analysing *Arabidopsis* RNA-seq data to quantify differential transcript abundance and expression.

Introduction

Alternative splicing (AS) plays a key regulatory role in the growth, development and behaviour of eukaryotic organisms. AS generates multiple transcript isoforms from a gene through variable selection of different splice sites in the precursor mRNA (Black, 2003; Stamm *et al.*, 2005; Nilsen & Graveley, 2010). The two main

consequences of AS are an increase in proteome complexity by generation of different protein isoforms often with different functionality (Black, 2003; Stamm *et al.*, 2005; Nilsen & Graveley, 2010) and the regulation of gene expression through degradation of specific transcripts by the nonsense-mediated decay (NMD) pathway (Nicholson & Mühlemann, 2010; Kalyna *et al.*, 2012; Schweingruber *et al.*, 2013).

In higher plants, AS has been implicated in a wide range of developmental and physiological processes (Syed *et al.*, 2012;

*These authors contributed equally to this work.

Carvalho *et al.*, 2013; Reddy *et al.*, 2013; Staiger & Brown, 2013). The functional importance of AS in plants has been illustrated in, for example, organ development, flowering time, and the circadian clock (Zhang & Mount, 2009; Sanchez *et al.*, 2010; Airoidi & Davies, 2012; James *et al.*, 2012; Jones *et al.*, 2012; Posé *et al.*, 2013), dark–light retrograde signalling from chloroplast to nucleus (Petrillo *et al.*, 2014), and zinc tolerance (Remy *et al.*, 2014). AS can be tissue-specific (Kriechbaumer *et al.*, 2012) or regulated by miRNAs and long noncoding RNAs (Jia & Rock, 2013; Bardou *et al.*, 2014). The estimated frequency of AS in plants has increased significantly over the last 10 years (Syed *et al.*, 2012). RNA sequencing (RNA-seq) analyses showed that in Arabidopsis at least 61% of intron-containing genes undergo AS (Filichkin *et al.*, 2010; Marquez *et al.*, 2012). RNA-seq permits the genome-wide identification of all transcript isoforms/splicing variants of a gene and the contribution that each transcript makes to expression. Currently transcript annotation in, for example, The Arabidopsis Information Resource (TAIR), is incomplete, and novel transcript variants continue to be identified in RNA-seq studies (Filichkin *et al.*, 2010; Marquez *et al.*, 2012). As the availability of RNA-seq data grows and AS becomes a routine part of expression analysis, the resolution of gene expression studies will increase and will require accurate methods to quantify transcript isoforms in RNA-seq. There is a range of available analysis tools that quantify transcripts from reads mapped to a genome or a transcriptome such as Tophat/CUFFLINKS (Trapnell *et al.*, 2009, 2010, 2013; Roberts *et al.*, 2011), RSEM (Li & Dewey, 2011; Li *et al.*, 2014), eXPRESS (Roberts & Pachter, 2013), Bayesemblem (Maretty *et al.*, 2014), and STRING TIE (Pertea *et al.*, 2015). A recent study compared different methods of analysing AS in plant RNA-seq data and showed variation in their ability to detect and quantify AS events with the accuracy of annotation having a major effect (Liu *et al.*, 2014).

Here, we describe the novel application of SAILFISH (Patro *et al.*, 2014) and SALMON (R. Patro *et al.*, unpublished) to Arabidopsis RNA-seq data. These related programmes are based on lightweight models of sequence alignment and efficient, parallel statistical inference algorithms. SAILFISH is a k -mer based method that uses lightweight algorithms to assign k -mers from RNA-seq reads to transcripts defined in a reference transcriptome (Patro *et al.*, 2014). Transcripts are quantified by counting the number of k -mers in RNA-seq reads and using an expectation-maximization algorithm to generate individual transcript abundances in transcripts per million (TPM) units (Patro *et al.*, 2014). SALMON is based on a novel lightweight alignment model that uses chains of maximal exact matches between sequencing fragments and reference transcripts to determine the potential origin of RNA-seq reads, and thus eliminates the dependence on a particular, predefined k -mer size required by methods such as SAILFISH. SALMON also relies on a novel streaming inference algorithm (an extension of stochastic collapsed variational Bayesian inference; Foulds *et al.*, 2013) to improve the accuracy of transcript abundance estimates while maintaining the fast speed and limited memory requirements of SAILFISH; it also produces abundance estimates in terms of TPM. In order to use SAILFISH and SALMON, we have developed a comprehensive Arabidopsis reference transcript dataset (AtRTD), and have generated quantitative TPM data from RNA-seq. We have

obtained high correlations between data from RNA-seq and from the high resolution reverse transcription polymerase chain reaction (HR RT-PCR) system (Simpson *et al.*, 2008) with SALMON giving the more accurate quantification. The AtRTD is a resource that will have immediate utility in analysing of Arabidopsis RNA-seq data to quantify differential transcript abundance using SAILFISH, SALMON or other programmes. This approach will be applicable to other plant species dependent on quality and depth of reference transcript dataset construction.

Materials and Methods

Construction of AtRTD

To construct the AtRTD, transcripts from The Arabidopsis Information Resource version 10 (TAIR10) (Lamesch *et al.*, 2012) and from the AS discovery RNA-seq analysis, which identified *c.* 50k novel transcripts (Marquez *et al.*, 2012), were merged. The AtRTD was refined and developed through iterative validation with HR RT-PCR data (see the HR RT-PCR subsection). Redundant transcripts were removed using available tools. First, to remove any confusion caused by differences in the coordinates for the same gene models in the two transcript annotation files, the start and end coordinates of the same gene models in TAIR10 and the AS discovery RNA-seq analysis (Marquez *et al.*, 2012) were compared. Where one was contained within another, the smaller one was removed; where models overlapped partially, the coordinates of the gene model in TAIR10 were retained. Second, the GTF file that describes all the transcript isoforms from the AS discovery RNA-seq analysis (Marquez *et al.*, 2012) was converted to GFF format using perl scripts (<http://search.cpan.org/~lds/GBrowse/bin/gtf2gff3.pl> version 0.1). The GFF transcript annotation files from the two sources described earlier were then sorted, checked and intron coordinates were extracted using GENOME TOOLS version 1.5.3 (Gremme *et al.*, 2013). Thus, when transcripts had the same intron coordinates but differed only in the length of their 5' or 3' untranslated regions (UTRs), the longest was retained. Similarly, redundant transcripts from single-exon genes with different lengths of 5' or 3' UTRs were removed. Finally, where transcript fragments occurred, probably due to low read coverage, but had the same intron coordinates of the longer or full-length transcripts, the fragments were removed. The FASTA file of the merged, nonredundant transcripts was generated using GFFREAD from the CUFFLINKS tool suite (Trapnell *et al.*, 2010). In addition, 33 novel transcripts of core clock genes identified previously (James *et al.*, 2012) were also added into the final transcript dataset. These transcripts were identified by HR RT-PCR; this illustrates that transcripts identified by other methods can be added to the AtRTD although we expect that the need for such manual curation will reduce as the AtRTD develops further. Thus, AtRTD represents a nonredundant, highly comprehensive reference transcript dataset that can be used with transcript quantification programmes. Here, the recently developed SAILFISH (k -mer = 30 nt) and SALMON programmes (Patro *et al.*, 2014; R. Patro, unpublished) were used in conjunction with the AtRTD to analyse ultra-deep RNA-seq data of

5-wk-old *Arabidopsis* plants grown at 20°C and then transferred to 4°C. Three biological samples were harvested at two time-points: dawn at 20°C (T1) and in the middle of the dark period four days after transfer to the low temperature (T2) for library preparation and sequencing. In total, *c.* 174 and 191 M 100 bp paired end reads were generated for T1 and T2, respectively.

HR RT-PCR

To validate the quantification of transcript isoforms, HR RT-PCR was performed on RNA from the same plant material as RNA-seq. Primer pairs covering 50 AS events in 29 genes, where the upstream primer was end-labelled with a fluorescent tag, were used in RT-PCR reactions with 24 cycles of PCR as described previously (Simpson *et al.*, 2008; James *et al.*, 2012; Kalyna *et al.*, 2012; Marquez *et al.*, 2012) and separated on an Illumina 3700 automatic DNA sequencing machine. The abundance of RT-PCR products was analysed with GENEMAPPER and splicing ratios were calculated from peak areas of each product. We analysed the RNA-seq data from the same regions and used the TPM values of the transcripts to calculate splicing ratios for comparison with those generated by HR RT-PCR, following a similar approach to Alamancos *et al.* (2015).

Results and Discussion

AtRTD was constructed by merging transcripts from TAIR10 and the AS discovery project (Marquez *et al.*, 2012). The latter study used a normalized library for RNA-seq to increase the depth of sequencing and significantly increased the number of transcript isoforms in *Arabidopsis* despite only using 10-d-old seedlings and flower tissue (Marquez *et al.*, 2012). The number of genes and transcripts contained in TAIR10, the AS discovery data and AtRTD are shown in Table 1. The gene number in TAIR10 and AtRTD differed by only 23 genes; the lower gene number in Marquez *et al.* (2012) reflects the number of expressed genes in two developmental stages. By contrast, the number of transcripts increased from 41 671 in TAIR10 (that also contains redundant transcripts which differ only by different lengths of 5' and 3' UTRs) to 74 216 in the merged and curated AtRTD dataset. The number of transcripts per gene, which reflects transcript isoform complexity, increased from 1.24 in TAIR10, to 2.40 and 2.21 in the AS discovery data and AtRTD, respectively (Table 1). Although the average transcripts per gene values are similar, the number of genes

Table 1 Number of *Arabidopsis thaliana* genes and transcripts in different datasets

	Number of genes	Number of transcripts	Average transcripts per gene
TAIR10	33 602	41 671 ^a	1.24
Marquez <i>et al.</i> (2012)	23 905	57 408 ^b	2.40
AtRTD v3	33 625	74 216 ^c	2.21

Note: TAIR10, The Arabidopsis Information Resource version 10; AtRTD, Arabidopsis reference transcript dataset. ^aContains redundant transcripts which differ only by different lengths of 5' and 3' UTRs. ^b*De novo* assembled transcripts defined by splice junctions. ^cMerged, nonredundant transcripts.

differs significantly (23 905 and 33 625, respectively). The increased transcript complexity in AtRTD compared with TAIR10 is also shown by the number of genes with higher numbers of transcripts (Fig. 1; Supporting Information Table S1). This is illustrated by the reduced number of genes with a single transcript in AtRTD (18 948) compared with TAIR10 (27 717). Thus, AtRTD represents a nonredundant transcript dataset that is highly enriched in AS transcripts. There are 14 499 intron-containing genes with more than one transcript suggesting that 63.22% of intron-containing genes undergo AS. It is, however, important to note that because not all developmental stages or environmental conditions are included, it is likely that AS transcripts are still not completely represented and new versions of AtRTD will be generated as other high quality RNA-seq data becomes available.

To demonstrate the utility of the AtRTD, we have used this set of transcripts to quantify transcripts in RNA-seq data with the SAILFISH and SALMON quantification tools. To validate the quantification of the resulting transcript abundances from RNA-seq, the TPM of individual transcripts for the genes used in HR RT-PCR were extracted from the RNA-seq data. Transcript structures were compared to the AS events covered by the primers in HR RT-PCR and used to calculate splicing ratios for each of the AS events in that region. The splicing ratio for this comparison is the percentage of transcripts with a particular AS event expressed as a function of the level of total transcripts (Fig. 2). Figure 2 shows histograms of splicing ratios of two gene/AS examples demonstrating the high degree of similarity between the RNA-seq SAILFISH and SALMON outputs and HR RT-PCR. *HEAT SHOCK FACTOR 3 (HSF3)* has three different transcripts resulting from AS in a 3'UTR intron: fully spliced, use of alternative 3' splice site which removes 8 nt from exon 3 and intron 2 retention (Fig. 2a). All three are relatively abundant transcripts and show small differences in splicing ratios at the different time-points. *RUBISCO ACTIVASE (RCA)* undergoes AS in intron 6 (Fig. 2b). An alternative 5' splice site within the intron adds 11 nt and changes the frame of the resulting protein to give different C-terminal sequences. Proteins from these two variants differ in length by 28 amino acids and contain eight or 36

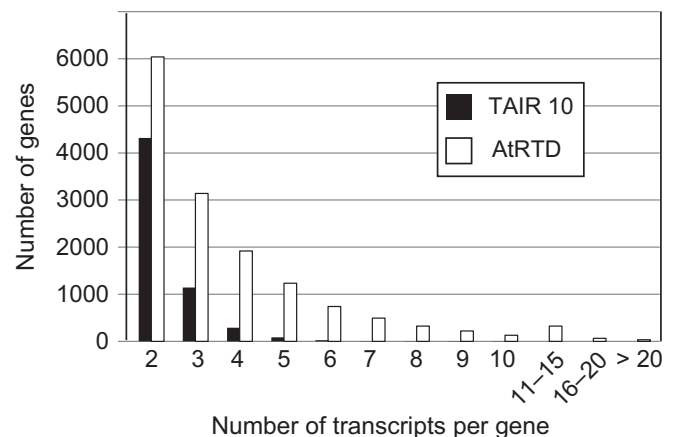


Fig. 1 Distribution of the number of transcripts per gene in The Arabidopsis Information Resource version 10 (TAIR10) and the Arabidopsis reference transcript dataset (AtRTD). The number of *Arabidopsis thaliana* genes (y-axis) containing two or more transcripts (x-axis) are shown.

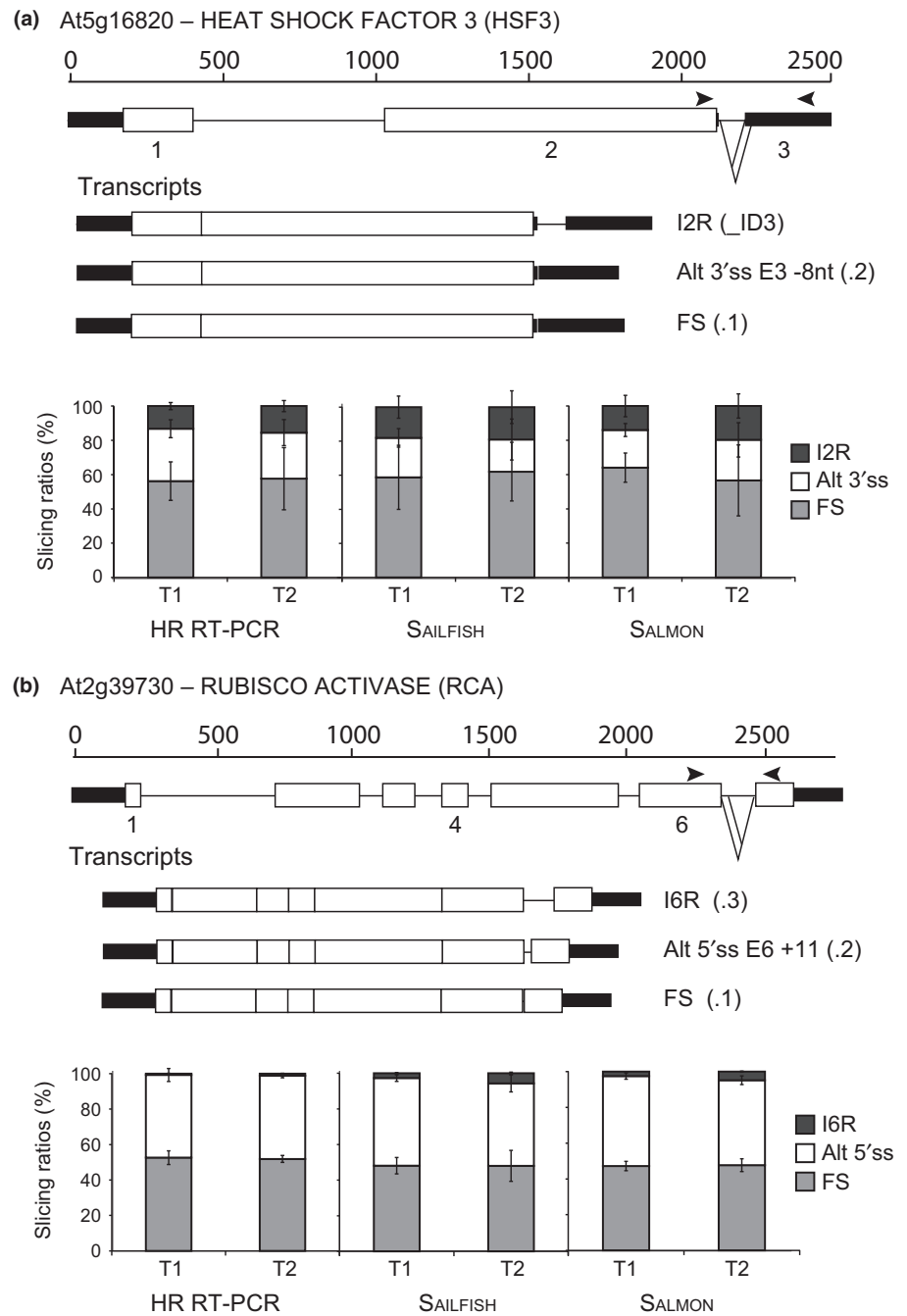


Fig. 2 *Arabidopsis thaliana* gene and transcript structures and histograms of transcript ratios from transcripts per million (TPM) generated by SAILFISH and SALMON with Arabidopsis reference transcript dataset (AtRTD) and from relative fluorescence units (RFUs) that measures peak areas from high resolution reverse transcription polymerase chain reaction (HR RT-PCR). (a) At5g16820, HEAT SHOCK FACTOR 3 (HSF3); (b) At2g39730, RUBISCO ACTIVASE (RCA). Transcripts are shown below the gene structure. Open boxes, exons; black rectangles, untranslated regions; thin lines, introns; diagonal lines, splicing events; arrowheads, approximate positions of primers used in HR RT-PCR. FS, fully spliced; E, exon; I2R/I6R, intron retention of introns 2 and 6 in (a) and (b), respectively; Alt 3'/5'ss, alternative (a) 3' and (b) 5' splice sites, respectively; T1 and T2, different time-points. Transcript variants from The Arabidopsis Information Resource (TAIR) and the alternative splicing (AS) discovery dataset (Marquez *et al.*, 2012) are indicated by .1, .2, or _ID3, respectively. Error bars represent the \pm standard deviation (SD) of three independent biological replicates.

different amino acids at their C-terminal ends. These examples demonstrate that RNA-seq detects multiple transcripts including transcripts which are present in low abundance (Fig. 2b, I6R), and distinguishes between transcripts differing by a small number of nucleotides (Fig. 2). To directly compare the output from SAILFISH and SALMON with HR RT-PCR, we analysed 50 AS events from 29 genes and three biological replicates of the two time-points (a total of 300 data points). As in some cases HR RT-PCR detected relatively low abundance AS transcripts which were not identified in RNA-seq, for this comparison we calculated a splicing ratio by comparing the abundance of the AS transcript to that of the fully spliced transcript (Fig. 3). For SAILFISH and SALMON, the Pearson's

correlation coefficient was 0.7044 and 0.9051, respectively, and the Spearman's rank correlation coefficient was 0.712 and 0.907, respectively (Fig. 3).

In this paper we demonstrate that the combination of the AtRTD (a comprehensive nonredundant reference transcript dataset for Arabidopsis) with SAILFISH or SALMON allows accurate estimation of individual transcript abundances. The novel AtRTD resource contains a significantly higher number of transcript isoforms than TAIR10 that is still widely used as a reference to analyse RNA-seq in Arabidopsis. A high degree of correlation between splicing ratios calculated from TPM from RNA-seq data and the HR RT-PCR was observed with SALMON

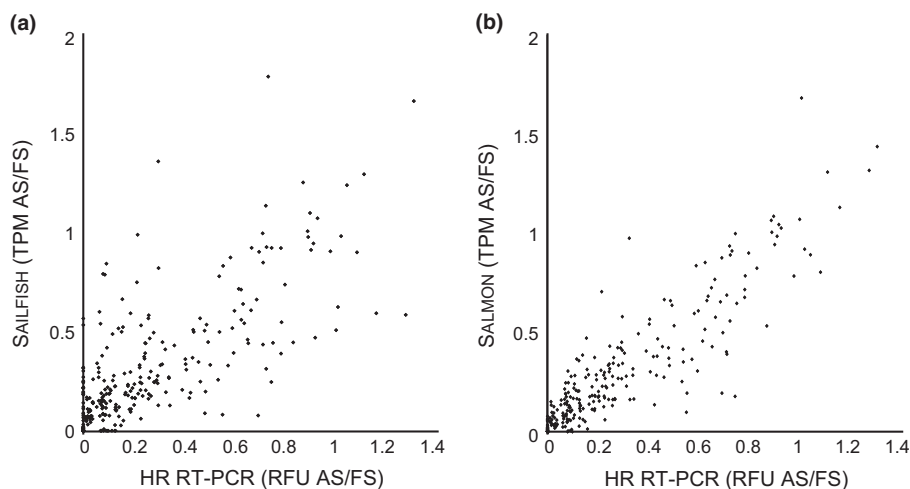


Fig. 3 Correlation of the splicing ratios calculated from the RNA-seq data and the high resolution reverse transcription polymerase chain reaction (HR RT-PCR). (a) SAILFISH, (b) SALMON. Splicing ratios for 50 alternative splicing events from 29 *Arabidopsis thaliana* genes (three biological replicates of the time points T1 and T2) generated 300 data points in total. AS, alternatively spliced; FS, fully spliced; TPM, transcripts per million; RFU, relative fluorescence unit. Pearson's correlation coefficient: SAILFISH = 0.7044, SALMON = 0.9051. Spearman's rank correlation coefficient: SAILFISH = 0.712, SALMON = 0.907.

outperforming SAILFISH. The HR RT-PCR system was used previously to validate RNA-seq data qualitatively (Marquez *et al.*, 2012) and to monitor changes in AS under different growth conditions and in a range of different mutants (Simpson *et al.*, 2008; Raczynska *et al.*, 2010; James *et al.*, 2012; Jones *et al.*, 2012; Kalyna *et al.*, 2012; Streitner *et al.*, 2012; Petrillo *et al.*, 2014) and here we demonstrate its utility in validating quantitative RNA-seq transcript data. RNA-seq is now widely used in transcriptome/expression analyses in plants to examine, for example, abiotic and biotic stress responses, development, light regulation and AS itself (e.g. Zhang *et al.*, 2010; Rühl *et al.*, 2012; Li *et al.*, 2013; Ding *et al.*, 2014; Thatcher *et al.*, 2014; Wu *et al.*, 2014; Mandadi & Scholthof, 2015). The AtRTD is freely available for download at <http://ics.hutton.ac.uk/atRTD/> and can now be used to analyse such *Arabidopsis* RNA-seq data.

Acknowledgements

This research was supported by funding from the Biotechnology and Biological Sciences Research Council (BBSRC) (BB/K006568/1 to J.W.S.B.; BB/K006835/1 to H.G.N.), the Scottish Government Rural and Environment Science and Analytical Services division (RESAS) and by the Austrian Science Fund (FWF) (P26333) to M.K. and (DK W1207) to A.B. The authors acknowledge the European Alternative Splicing Network of Excellence (EURASNET), LSHG-CT-2005-518238 for catalysing important collaborations. The authors thank Janet Laird (University of Glasgow) and Linda Milne (James Hutton Institute) for technical assistance.

References

- Airoidi CA, Davies B. 2012. Gene duplication and the evolution of plant MADS-box transcription factors. *Journal of Genetics and Genomics* 39: 157–165.
- Alamancos GP, Pagès A, Trincado JL, Bellora N, Eyra E. 2015. Leveraging transcript quantification for fast computation of alternative splicing profiles. *bioRxiv*. doi: 10.1101/008763.

- Bardou F, Ariel F, Simpson CG, Romero-Barríos N, Laporte P, Balzerque S, Brown JW, Crespi M. 2014. Long noncoding RNA modulates alternative splicing regulators in *Arabidopsis*. *Developmental Cell* 30: 166–176.
- Black DL. 2003. Mechanisms of alternative pre-messenger RNA splicing. *Annual Review of Biochemistry* 72: 291–336.
- Carvalho RF, Feijão CV, Duque P. 2013. On the physiological significance of alternative splicing events in higher plants. *Protoplasma* 250: 639–650.
- Ding F, Cui P, Wang Z, Zhang S, Ali S, Xiong L. 2014. Genome-wide analysis of alternative splicing of pre-mRNA under salt stress in *Arabidopsis*. *BMC Genomics* 15: 431.
- Filichkin SA, Priest HD, Givan SA, Shen R, Bryant DW, Fox SE, Wong WK, Mockler TC. 2010. Genome-wide mapping of alternative splicing in *Arabidopsis thaliana*. *Genome Research* 20: 45–58.
- Foulds J, Boyles L, DuBois C, Smyth P, Welling M. 2013. Stochastic collapsed variational Bayesian inference for latent Dirichlet allocation. *19th ACM SIGKDD International Conference on Knowledge Discovery and Data Mining*. New York, NY, USA: ACM, 446–454.
- Gremme G, Steinbiss S, Kurtz S. 2013. GenomeTools: a comprehensive software library for efficient processing of structured genome annotations. *IEEE/ACM Transactions on Computational Biology and Bioinformatics* 10: 645–656.
- James AB, Syed NH, Bordage S, Marshall J, Nimmo GA, Jenkins GI, Herzyk P, Brown JWS, Nimmo HG. 2012. Alternative splicing mediates responses of the *Arabidopsis* circadian clock to temperature changes. *Plant Cell* 24: 961–981.
- Jia F, Rock CD. 2013. *MIR846* and *MIR842* comprise a cistronic *MIRNA* pair that is regulated by abscisic acid by alternative splicing in roots of *Arabidopsis*. *Plant Molecular Biology* 81: 447–460.
- Jones MA, Williams BA, McNicol J, Simpson CG, Brown JWS, Harmer SL. 2012. Mutation of *Arabidopsis* *SPLICEOSOMAL TIMEKEEPER LOCUS1* causes circadian clock defects. *Plant Cell* 24: 4066–4082.
- Kalyna M, Simpson CG, Syed NH, Lewandowska D, Marquez Y, Kusenda B, Marshall J, Fuller J, Cardle L, McNicol J *et al.* 2012. Alternative splicing and nonsense-mediated decay modulate expression of important regulatory genes in *Arabidopsis*. *Nucleic Acids Research* 40: 2454–2469.
- Kriechbaumer V, Wang P, Hawes C, Abell BM. 2012. Alternative splicing of the auxin biosynthesis gene *YUCCA4* determines its subcellular compartmentation. *Plant Journal* 70: 292–302.
- Lamesch P, Berardini TZ, Li D, Swarbreck D, Wilks C, Sasidharan R, Muller R, Dreher K, Alexander DL, Garcia-Hernandez M *et al.* 2012. The *Arabidopsis* Information Resource (TAIR): improved gene annotation and new tools. *Nucleic Acids Research* 40(Database issue): D1202–D1210.
- Li B, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12: 323.
- Li B, Fillmore N, Bai Y, Collins M, Thomson JA, Stewart R, Dewey CN. 2014. Evaluation of *de novo* transcriptome assemblies from RNA-Seq data. *Genome Biology* 15: 553.

- Li W, Lin WD, Ray P, Lan P, Schmidt W. 2013. Genome-wide detection of condition-sensitive alternative splicing in *Arabidopsis* roots. *Plant Physiology* **162**: 1750–1763.
- Liu R, Loraine AE, Dickerson JA. 2014. Comparisons of computational methods for differential alternative splicing detection using RNA-seq in plant systems. *BMC Bioinformatics* **15**: 364.
- Mandadi KK, Scholthof KB. 2015. Genome-wide analysis of alternative splicing landscapes modulated during plant–virus interactions in *Brachypodium distachyon*. *Plant Cell* **27**: 71–85.
- Marettty L, Sibbesen JA, Krogh A. 2014. Bayesian transcriptome assembly. *Genome Biology* **15**: 501.
- Marquez Y, Brown JW, Simpson CG, Barta A, Kalyna M. 2012. Transcriptome survey reveals increased complexity of the alternative splicing landscape in *Arabidopsis*. *Genome Research* **22**: 1184–1195.
- Nicholson P, Mühlemann O. 2010. Cutting the nonsense: the degradation of PTC-containing mRNAs. *Biochemical Society Transactions* **38**: 1615–1620.
- Nilsen TW, Graveley BR. 2010. Expansion of the eukaryotic proteome by alternative splicing. *Nature* **463**: 457–463.
- Patro R, Mount SM, Kingsford C. 2014. Sailfish enables alignment-free isoform quantification from RNA-seq reads using lightweight algorithms. *Nature Biotechnology* **32**: 462–464.
- Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. 2015. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nature Biotechnology* **33**: 290–295.
- Petrillo E, Godoy Herz MA, Fuchs A, Reifer D, Fuller J, Yanovsky MJ, Simpson CG, Brown JW, Barta A, Kalyna M *et al.* 2014. A chloroplast retrograde signal regulates nuclear alternative splicing. *Science* **344**: 427–430.
- Posé D, Verhage L, Ott F, Yant L, Mathieu J, Angenent GC, Immink RGH, Schmid M. 2013. Temperature-dependent regulation of flowering by antagonistic FLM variants. *Nature* **503**: 414–417.
- Raczynska KD, Simpson CG, Ciesiolka A, Szewc L, Lewandowska D, McNicol J, Szwejkowska-Kulinska Z, Brown JW, Jarmolowski A. 2010. Involvement of the nuclear cap-binding protein complex in alternative splicing in *Arabidopsis thaliana*. *Nucleic Acids Research* **38**: 265–278.
- Reddy AS, Marquez Y, Kalyna M, Barta A. 2013. Complexity of the alternative splicing landscape in plants. *Plant Cell* **25**: 3657–3683.
- Remy E, Cabrito TR, Batista RA, Hussein MA, Teixeira MC, Athanasiadis A, Sá-Correia I, Duque P. 2014. Intron retention in the 5'UTR of the novel ZIF2 transporter enhances translation to promote zinc tolerance in *Arabidopsis*. *PLoS Genetics* **10**: e1004375.
- Roberts A, Pachter L. 2013. Streaming fragment assignment for real-time analysis of sequencing experiments. *Nature Methods* **10**: 71–73.
- Roberts A, Pimentel H, Trapnell C, Pachter L. 2011. Identification of novel transcripts in annotated genomes using RNA-Seq. *Bioinformatics* **27**: 2325–2329.
- Rühl C, Stauffer E, Kahles A, Wagner G, Drechsel G, Ratsch G, Wachter A. 2012. Polypyrimidine tract binding protein homologs from *Arabidopsis* are key regulators of alternative splicing with implications in fundamental developmental processes. *Plant Cell* **24**: 4360–4375.
- Sanchez SE, Petrillo E, Beckwith EJ, Zhang X, Rugnone ML, Hernando CE, Cuevas JC, Godoy Herz MA, Depetris-Chauvin A, Simpson CG *et al.* 2010. A methyl transferase links the circadian clock to the regulation of alternative splicing. *Nature* **468**: 112–116.
- Schweingruber C, Rufener SC, Zünd D, Yamashita A, Mühlemann O. 2013. Nonsense-mediated mRNA decay – mechanisms of substrate mRNA recognition and degradation in mammalian cells. *Biochimica et Biophysica Acta* **1829**: 612–623.
- Simpson CG, Fuller J, Maronova M, Kalyna M, Davidson D, McNicol J, Barta A, Brown JWS. 2008. Monitoring changes in alternative precursor messenger RNA splicing in multiple gene transcripts. *Plant Journal* **53**: 1035–1048.
- Staiger D, Brown JWS. 2013. Alternative splicing at the intersection of biological timing, development, and stress responses. *Plant Cell* **25**: 3640–3656.
- Stamm S, Ben-Ari S, Rafalska I, Tang Y, Zhang Z, Toiber D, Thanaraj TA, Soreq H. 2005. Function of alternative splicing. *Gene* **344**: 1–20.
- Streitner C, Köster T, Simpson CG, Shaw P, Danisman S, Brown JWS, Staiger D. 2012. An hnRNP-like RNA-binding protein affects alternative splicing by *in vivo* interaction with transcripts in *Arabidopsis thaliana*. *Nucleic Acids Research* **40**: 11240–11255.
- Syed NH, Kalyna M, Marquez Y, Barta A, Brown JWS. 2012. Alternative splicing in plants – coming of age. *Trends in Plant Science* **17**: 616–623.
- Thatcher SR, Zhou W, Leonard A, Wang B, Beatty M, Zastrow-Hayes G, Zhao X, Baumgarten A, Li B. 2014. Genome-wide analysis of alternative splicing in *Zea mays*: landscape and genetic regulation. *Plant Cell* **26**: 3471–3487.
- Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L. 2013. Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nature Biotechnology* **31**: 46–53.
- Trapnell C, Pachter L, Salzberg SL. 2009. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* **25**: 1105–1111.
- Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature Biotechnology* **28**: 511–515.
- Wu HP, Su YS, Chen HC, Chen YR, Wu CC, Lin WD, Tu SL. 2014. Genome-wide analysis of light-regulated alternative splicing mediated by photoreceptors in *Physcomitrella patens*. *Genome Biology* **15**: R10.
- Zhang G, Guo G, Hu X, Zhang Y, Li Q, Li R, Zhuang R, Lu Z, He Z, Fang X *et al.* 2010. Deep RNA sequencing at single base-pair resolution reveals high complexity of the rice transcriptome. *Genome Research* **20**: 646–654.
- Zhang XN, Mount SM. 2009. Two alternatively spliced isoforms of the *Arabidopsis thaliana* SR45 protein have distinct roles during normal plant development. *Plant Physiology* **150**: 1450–1458.

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Transcript complexity of AtRTD: distribution of the number of transcripts per gene

Please note: Wiley Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.