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Abstract

Assembly of the barley genome and extensive use of RNA-seq has resulted in an abundance of gene expression data and the recognition of wide scale production of alternatively spliced transcripts. Here, we describe in detail a high-resolution reverse transcription-PCR based panel (HR RT-PCR) that confirms the accuracy of alternatively spliced transcripts from RNA-seq and allows quantification of changes in the proportion of splice isoforms between different experimental conditions, time points, tissues, genotypes, ecotypes and treatments. By validating a selection of barley genes, use of the panel gives confidence or otherwise to the genome-wide global changes in alternatively spliced transcripts reported by RNA-seq. This simple assay can readily be applied to perform detailed transcript isoform analysis for any gene in any species.

Key words: HR RT-PCR; alternative splicing, RNA-seq

1. Introduction

Improving barley performance in the field depends on understanding how traits develop and the underlying regulation of expressed genes. The formation of alternative transcripts by the process of alternative splicing (AS) has a strategic role in the multiple layers of transcriptional, mRNA stability and co-ordinated post-transcriptional networks that function to deliver gene products at the right time and place **(1-4)**. AS increases the protein coding capacity of eukaryote genomes and leads to the regulation and fine tuning of gene expression **(2, 5-6)**. During AS, different splice site choice results in different messenger RNA (mRNA) isoforms: the size of exons can vary due to the use of alternative 5´or 3´splice

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sites; exons can be alternatively included or excluded, termed exon skipping; and introns can remain in the mRNA, designated intron retention. The selection of alternative splice sites is dictated by *cis*-acting motifs located in the precursor mRNAs (pre-mRNAs) that serve as recognition sites for RNA-binding proteins and accessory factors involved in the splicing process. Variation in AS is often tissue-specific and the pattern of AS can be developmentally and environmentally regulated **(4-5,7-8)**. AS produces alternative proteins that vary, for example, in their function, their interaction with other proteins and/or in their subcellular localisation. Furthermore, alternative splice isoforms can be recognized as "aberrant" and degraded by nonsense mediated decay (NMD), ultimately changing transcript abundance **(2)**.

Large scale sequencing of multiple RNA samples by next generation sequencing (NGS) technologies allows global *de novo* detection of individual gene transcript variants and the potential to quantify changes in transcript variant abundances. Using NGS across a range of eight different barley tissues, 55% of genes had alternative transcripts **(9)**. In germinating seed of four different barley varieties, AS was found in 14-20% of intron-containing genes, including those involved in cell wall polysaccharide metabolism **(10-11)**. To support and validate gene transcript changes identified by NGS, we have used a medium-throughput AS panel based on reverse transcription-PCR and separation of fluorescently labeled amplicons by capillary sequencing. High resolution RT-PCR has the power to resolve products that differ by a single base pair and detects statistically significant AS changes between different samples **(12)**. The panel was originally developed for Arabidopsis and has been widely used to demonstrate AS in natural plant variants, different plant organs; in plants grown under different conditions and in genes encoding core components of the circadian clock **(13-15)**. Moreover, the analysis of mutants defective in candidate splicing regulators such as serine-arginine rich proteins or the subunits of the cap-binding complex, as well as transgenic plants overexpressing hnRNP (heterogeneous nuclear ribonucleoproteins) proteins has demonstrated the global impact of these regulators on plant AS **(12, 16-18)**. The HR RT-PCR procedure is highly transferable to different genes and plant species. In barley, it has recently been used to show conserved temperature responsive alternatively spliced isoform switching in circadian clock genes between barley and Arabidopsis **(19)**.

We have assembled a panel of primers specific to selected barley genes that produce amplicons across barley AS events. We used these to confirm the accuracy of predicted AS events from the RNA-seq assembly and to determine the proportions of alternative transcripts produced.

2. Materials

2.1 RNA isolation

Qiagen RNeasy Mini Kit (50)

Promega RQ1 RNase-Free DNase.

Thermo Scientific, NanoDrop 2000 UV-Vis Spectrophotometer.

TE: 10 mM Tris and 1 mM EDTA, pH 8 HCl.

RNasin.

Phenol/Chloroform: Dissolve 250 g phenol with 100 ml Tris HCl pH 8 at 40°C for 1 hour. Add an equal volume of chloroform, mix and store at 4°C in a dark bottle.

Ethanol/Sodium Acetate pH 4.8 mix: Mix 19 Vol 100% Ethanol with 1 Vol 3M Sodium acetate pH 4.8.

2.2 cDNA synthesis

Clontech, RNA to cDNA EcoDry double primed premix

2.3 PCR Reagents

Taq DNA polymerase and 10 x Buffer.

dNTP's – Dilute 100 mM stock of each deoxynucleotide (dATP, dCTP, dGTP and dTTP) to 20 mM. Mix each nucleotide together with sterile distilled water to produce a 1.25 mM working dNTP stock.

Primers to detect AS events – Dilute 100 µM stock to 20 µM.

PCR plates (Thermo-Fast 96, Semi-skirted.)

2.4 Product separation

500 LIZ Size standard (ABI) for reproducible sizing of RT-PCR fragments. The 500 marker contains 16 single-stranded labeled fragments of different lengths

Hi Di Formamide (ABI)

2.5. Software

Applied Biosystems. Genemapper v3.7 or above.

Microsoft Excel

Genstat or R statistical analysis software.

3. Protocols

3.1 Gene selection

Any gene of interest that shows AS may be tested for changes in splicing. The AS panel will increase as more alternatively spliced genes are identified and tested. Initially, we selected 94 AS events from an NGS RNA-seq experiment of 8 different organs. Each AS event was supported by at least 100 splice junction reads and represented the major types of AS (exon skipping; alternative 5' and 3' splice sites; and intron retention). At present, the barley AS panel consists of 215 genes covering 250 AS events. For our AS analysis, we included the AS event found in barley rubisco activase as an AS control. This was the first plant gene identified to show AS, is highly expressed in most photosynthetic tissues and produces two transcripts through AS that are often found in an approximately 1:1 ratio **(20)**. Protein phosphatase 2A subunit A2 (MLOC_2967; HORVU5Hr1G051850) was also included in our analyses as a transcriptional control to normalise transcription to steady state levels, if required **(19)**.

3.2 Primer design

Primers are designed by selection of sequences within exon sequences upstream and downstream of the AS event(s). To study intron retention events, primers are designed across a constitutively spliced intron and the alternatively retained intron (*see* Note 1). This avoids false positive intron retention results that may occur due to contaminating DNA. Selected primers are usually 19-25 nt long, about 50% GC and produce spliced PCR products that are predicted to be less than 750 bp in length, which are readily detectable in the sequencing run (ABI3730) using the 500 bp marker. Both 5' and 3' primers contain one or two G or C nucleotides at their 5' and 3' ends to avoid primer dimerisation. Primers are BLAST screened against the barley genome to avoid primers that have a perfect match with other regions in the genome. The 5' forward primer is labelled at its 5' end with a 6-FAM (6-Carboxyfluorescein) fluorescent dye (*see* Note 2).

3.3 RNA Extraction.

1. Plant material (*see* Note 3)– Extract total RNA from up to 100 mg of any selected barley tissue using available RNA extraction kits. We use the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions (see Qiagen RNeasy manual).

2. Determine RNA concentrations by Nanodrop (Thermo Scientific).

3. Most RNA extraction kits leave very little contaminating DNA, but nevertheless can be picked up by sensitive PCR. Our AS analysis does not usually take into consideration any unspliced RNA products that will produce the same PCR product size as contaminating DNA. If the AS analysis needs to consider unspliced products, RNA preparations are further treated with RQ1 DNase to remove remaining DNA. A maximum of 50 µg of RNA (50 µl) is

added to TE, 10 mM MgCl$_2$, 100U RNasin and 10U of RQ1 DNase in a final volume of 100 µl. Incubate the reaction at 37°C for 20 mins and terminate the reaction by adding 1/50th volume of 0.5 M EDTA pH 8 and 1/50th volume 10% (w/v) SDS. Extract RNA with an equal volume of Phenol/Chloroform pH 8 mix and precipate with 2.5 vols of Ethanol/Sodium acetate pH 4.8 held at -20°C. After pelleting by centrifugation, the RNA pellets are washed with 70% ethanol at -20°C, air dried and resuspended at a concentration of 1 µg/µl (*see* Note 4).

3.4 1st Strand cDNA Synthesis.

1. Add 5 µg of total RNA (enough for 100 PCR reactions, i.e. one 96 well-plate) to sterile distilled water to a volume of 20 µl.

2. Transfer the sample to 1 microfuge tube supplied which contains the "RNA to cDNA EcoDry double primed" first strand cDNA synthesis bead and leave at room temperature for 1 min (*see* Note 5).

3. Gently mix the sample by pipetting, spin briefly in a microfuge to collect the sample and incubate at 37ºC for 1 hour then at 70°C for 10 min. Add sterile distilled water to give a final volume of 100 µl.

3.5 PCR.

1. For each 25 µl PCR reaction the following is prepared (make a mastermix).

	x 1	x 100
10 x buffer	2.5 µl	250 µl
1.25 mM dNTP's (200 µM*)	4 µl	400 µl
Taq DNA polymerase	0.125 µl	12.5 µl
SDW	16.375 µl	1637.5µl

   * 200 µM is the final dNTP concentration.

2. For a 96-well plate reaction, add the complete first strand reaction mix to the 100 x PCR reaction mix. Add 24 µl of the resulting mix to each well of a 96-well plate containing 1 µl of the 96 different forward and reverse gene-specific primer pairs (400 nM per primer per reaction). This gives a total PCR reaction volume of 25 µl in each well.

3. Mix the samples by vortexing, spin briefly to collect the samples on the bottom of the well and place on a PCR machine (Perkin Elmer 9700) using the following cycle program:

1 cycle   94 ºC – 2 min

24 cycles   94ºC – 15 s

(*see* Note 6) 50ºC – 30 s

                70 ºC – 1 min

1 cycle   70ºC – 10 min

Store at 4ºC until ready to use.

3.6 Separation and analysis of the spliced products

1. Mix the labelled RT-PCR products from the RT-PCR reactions with Hi Di Formamide (Applied Biosystems) and the 500 LIZ labelled size marker (Applied Biosystems) (*see* Note 7). For the 96 reactions in the 96-well plate, prepare the following mix:

	x 1	x 100
500 LIZ Size standard	0.05 µl	5 µl
Hi Di Formamide	8.95 µl	895 µl

2. Aliquot 9µl of the mix into each well of a 96 well plate and add 1 µl of each RT-PCR reaction. Store the remaining sample at -20 ºC for downstream cloning and sequencing.

3. Inject samples, separate by capillary electrophoresis and detect on an ABI3730 DNA Analyzer (Life Technologies) or equivalent. Set up the platform for fragment analysis using a 36 cm capillary array, POP7 polymer and dye filter set G5. Run samples containing the LIZ 500 marker using the manufacturer's 'GeneMapper36_POP7' Run Module (run time 1200s, run voltage 15 Kv). Subsequently, the peak (RT-PCR product) sizes and areas are calculated and analysed with Life Technologies GeneMapper v3.7 (*see* Note 8).

4. RT-PCR products are accurately identified with ± 1-2 bp resolution. Extract the relative fluorescent peak areas for RT-PCR products with expected sizes for the alternatively spliced products and tabulate in Microsoft Excel (Table 1) (*see* Note 9).

3.7 Basic statistics

1. Calculate the relative proportion of the transcripts in the different AS events by dividing the value for each alternatively spliced product by the sum of the values for all the spliced products of that event.

2. For an accurate statistical measurement of AS proportions, three biological repeats are routinely performed for all experiments. Mean AS proportions with standard deviations and standard errors are calculated for the three separate biological repetitions (*see* Note 10).

3. For each alternatively spliced transcript, ANalysis Of VAriance (ANOVA) is used in turn to compare all the organs after an angular transformation of the individual AS proportions (*see* Note 11). In this case, analysis was performed on the arcsine scale and p-values are not adjusted for multiple comparisons (as an overall ANOVA p-value ≤0.001 is considered highly significant). The p-values are obtained from t-tests that are based on the residual variance estimate from all five samples tested. ANOVA assumes a completely randomised experimental design. AS events with significant variation (p=≤0.05) are routinely selected (*see* Note 12).

4. Example

A HR RT-PCR analysis is shown for a single primer pair that covers an AS event discovered in a barley clathrin adaptor complex subunit protein gene (MLOC_54446; HORVU5Hr1G027080) with similarity to the Arabidopsis gene At2g20790 (Fig 1a). This is one example from many hundreds of genes that can be examined simultaneously using the HR RT-PCR panel. It highlights the resolution obtained by this method and quantification of the proportions of the alternative transcripts.

RNA-seq analysis identified the alternative 5' splice site in this gene at intron 3, leading to alternative transcripts that differ by only 5 nt (Fig 1b). The shorter transcript using the distal 5' splice site produces a transcript that can be translated into a protein that matches the clathrin adaptor complex subunit protein. The longer transcript leads to a frame shift, introduces a premature termination codon and will result in a severely truncated protein.

HR RT-PCR validated the two HR RT-PCR products as 174 and 179 bp in length (Fig. 1c). RNA was extracted from five barley organs which included: whole developing inflorescence tissue 30 (INF1) and 50 (INF2) days after planting; leaf tissue, 17 days after planting (LEA); mesocotyl and seminal root tissue, 4 days after germination (EMB); and stem at the third internode, 42 days after planting (NOD) **(9)**. This was followed by HR RT-PCR and capillary electrophoresis to identify the different transcripts. Data collected on RT-PCR product length and peak area was extracted and tabulated for the three repeats and the proportions determined (Table 1). Mean values of the proportions were determined over the three repeats, followed by standard deviations and standard errors (Table 1). A graph of the data shows the proportional changes in alternative 5' splice site selection in the five different barley organs (Fig 1d). Pairwise ANOVA between the five barley organs identified the tissues that showed significant changes in alternative 5' splice site selection in this gene (Table 2). In this example, INF1 and NOD tissues have identical AS proportions and, therefore, show no

significant differences. Comparison of LEA with INF1 and NOD showed a significant change in AS to p=≤0.05. The remaining comparisons were all significant to p=≤0.01 highlighting the variation in AS that occurs in different barley organs. The overall conclusion from the data is that the shorter transcript, which codes for the functional protein, is more prevalent in leaf and embryo organs compared to the internode and inflorescence organs.

There are many different alogorithms to assemble and quantify individual transcripts based on read numbers from RNA-seq data that can be used to determine proportions of AS **(21)**. Computational systematic analysis of alternative splicing in large data sets can further be determined by calculating inclusion levels of alternative splicing events using algorithms as described for SUPPA **(22)**. These AS proportions can be directly compared with the alternative splice proportions derived from HR RT-PCR. In this simple example, AS proportions between the five different barley organs were highly comparable to RNA-seq data between the assays with a Pearson correlation value of 0.971 (Fig. 1e). This is not always the case and their are many examples where correlation is poor between the two methods. Different RNA-seq algorithms mis-map reads, mis-assemble transcripts and generate redundancy which affects accurate quantification. This has recently been addressed in Arabidopsis by quantifying alternative transcripts against a reference transcript database that contains only well-supported transcripts and a similar approach is underway for barley **(23,24)**. The HR RT-PCR method described here will be important in the development of the reference transcript database for barley and to validate quantification of individual transcripts produced by different RNA-seq analysis methods.

5. Notes

1. PCR is very sensitive and despite careful removal of contaminating DNA, enough DNA may remain to produce products that are indistinguishable from unspliced pre-mRNA. To overcome this, retained intron event primers are designed across a constitutively spliced intron upstream or downstream of the retained intron. Retained intron spliced products are therefore easily distinguished from the DNA-derived products, which would contain both introns.

2. Different fluorescent standard dye sets are available for genotyping applications. We have successfully used 6-FAM labelled primers in association with size markers labelled with ROX (6-Carboxyl-X-Rhodamine) or the proprietary LIZ label. As these are used as size markers, we avoid labelling primers with these fluorescent labels. We have further attempted to multiplex AS analysis using HEX (6-carboxy-1,4-dichloro-2',4',5',7'-tetra-chlorofluorescein) labelled primers. Depending on the amounts of transcripts made, we found overlap in the emission spectra between 6-FAM and HEX labelled primers, which led to the presence of

unexpected peaks in the other labelled RT-PCR products and confused downstream analysis. We have returned to single fluorescent dye label analysis.

3. AS is affected at different developmental stages and tissues. It is also affected by environmental abiotic and biotic stresses, and circadian times. High-resolution RT-PCR AS analysis is highly sensitive to these changes. It is therefore important that biological repeat plants including control plants are grown at the same time and in the same conditions with the exception of the condition tested.

4. In some cases, DNA may still remain after a single DNase treatment. A second DNase treatment will remove any remaining DNA.

5. The RNA to cDNA EcoDry Double primed cDNA synthesis beads contain both oligo dT and random hexamers. We have found an improvement in RT-PCR peaks using this double primed system.

6. A total of 24 PCR cycles were selected on the basis that PCR is in the logarithmic phase of amplification and allows us to screen both highly and more poorly transcribed genes. We previously tested the variation in AS results in technical replicates and the majority showed less than 1% variability with a small number reaching up to 3%. We set a value slightly higher at 5% variation as a cut-off value for statistically significant changes in AS (*see* Note 12). Samples taken at > 24 cycles showed that the more highly expressed genes were moving out of the exponential phase of PCR amplification (**25**).

7. We routinely use the 500 LIZ Size standard to calculate RT-PCR product sizes. For RT-PCR products greater than 750 bp the 1200 LIZ Size standard can be used. 1 µl of each reaction is mixed with 0.5 µl of the standard and 8.5 µl of Hi Di Formamide. Samples containing the LIZ 1200 marker are run using the '3730_36cm_POP7_GS1200Lizv2_1' Run Module (run time 6000s, run voltage 6.1 Kv).

8. Genmapper software is routinely updated. Check Life Technologies website for updates (https://www.lifetechnologies.com/uk/en/home.html).

9. The nature of AS means that you may obtain expected peaks (RT-PCR products) that vary substantially in their peak areas. The Genemapper software may be set to select a minimum peak size and reduce the number of small background peak areas that may be extracted and tabulated. However, we set the peak level low (200 relative fluorescent units) to capture as many of the alternatively spliced products as possible. Background peaks are therefore removed from the analysis.

10. The HR RT-PCR AS analysis measures changes in the proportions of the different alternative spliced transcripts found. Although, between the repeats, there may be apparent changes in the overall amounts of transcript produced, the proportion of the different alternatively spliced transcripts is maintained.

11. An angular transformation is often used with proportions to bring the data closer to a normal distribution. It is performed on the raw proportions before any statistical analysis is done. If p is the proportion then the transformation is $x = \arcsin(\sqrt{p})$.

12. In a number of cases where there is very little variation between the repeats, the standard errors may be very low and even small changes in the proportion of splicing may be identified as statistically significant with $p \leq 0.05$. We therefore, select examples that are statisticaly significant and show > 5% splicing change (*see* Note 6).

Acknowledgements

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Table 1. Extracted RT-PCR product length and peak areas for a single primer pair across five barley organs.

Sample File Name	Length Detected (bp)	Peak Area (RFU)	Proportion	Mean of 3 repeats (Sample File Reps 1-3)	SD	SE
INF1						
110_Inf1_Rep1	174.12	21750	0.61	0.58	0.0235	0.0136
110_Inf1_Rep1	179.07	13995	0.39	0.42	0.0235	0.0136
110_Inf1_Rep2	174.13	7662	0.56			
110_Inf1_Rep2	179.08	5906	0.44			
110_Inf1_Rep3	174.17	10550	0.57			
110_Inf1_Rep3	179.14	7909	0.43			
INF2						
110_Inf2_Rep1	174.52	9259	0.39	0.45	0.0546	0.0315
110_Inf2_Rep1	179.52	14374	0.61	0.55	0.0546	0.0315
110_Inf2_Rep2	174.57	8823	0.49			
110_Inf2_Rep2	179.67	9055	0.51			
110_Inf2_Rep3	174.71	9757	0.48			
110_Inf2_Rep3	179.81	10689	0.52			
LEA						
110_Lea_Rep1	174.45	5613	0.68	0.66	0.0268	0.0155
110_Lea_Rep1	179.48	2611	0.32	0.34	0.0268	0.0155
110_Lea_Rep2	174.42	2702	0.66			
110_Lea_Rep2	179.39	1417	0.34			
110_Lea_Rep3	174.52	2854	0.63			
110_Lea_Rep3	179.52	1684	0.37			
EMB						
110_Emb_Rep1	174.05	20120	0.81	0.79	0.0174	0.0100
110_Emb_Rep1	179.17	4675	0.19	0.21	0.0174	0.0100
110_Emb_Rep2	174.08	21418	0.79			
110_Emb_Rep2	179.07	5795	0.21			
110_Emb_Rep3	174.02	15452	0.78			
110_Emb_Rep3	179.20	4415	0.22			
NOD						
110_Nod_Rep1	174.13	4083	0.63	0.58	0.0470	0.0271
110_Nod_Rep1	179.24	2411	0.37	0.42	0.0470	0.0271
110_Nod_Rep2	174.19	4444	0.57			
110_Nod_Rep2	179.31	3287	0.43			

110_Nod_Rep3	174.14	4362	0.54		
110_Nod_Rep3	179.24	3790	0.46		

Sample file name indicates primer number, barley organ and repeat number.

SD = Standard deviation = $\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2 /(n-1)}$ where n is the number of repeats, x_i is the individual value and $\bar{x}$ is the mean of the repeats.
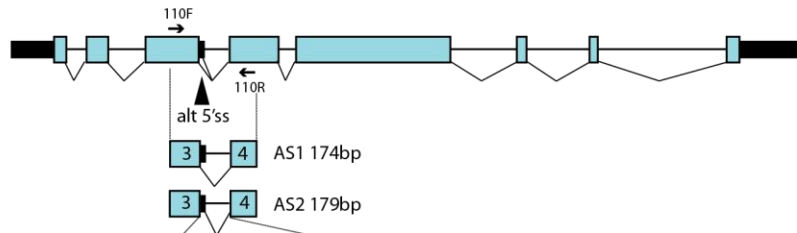
SE = Standard error of mean. SE = SD /√n where n is the number of repeats.
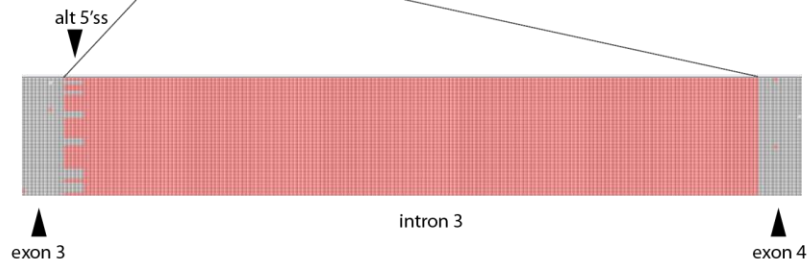
Table 2 Analysis of variance between barley organs

	INF1	INF2	LEA	EMB
INF2	0.001959			
LEA	0.027415	0.000051		
EMB	0.000018	0.000000	0.000515	
NOD	0.995754	0.001942	0.027673	0.000018

Values given as p values = the significance value from the t-test analysis of variance.
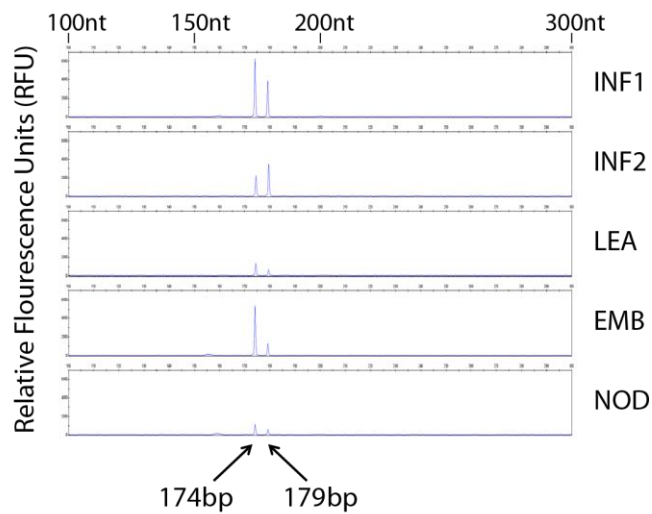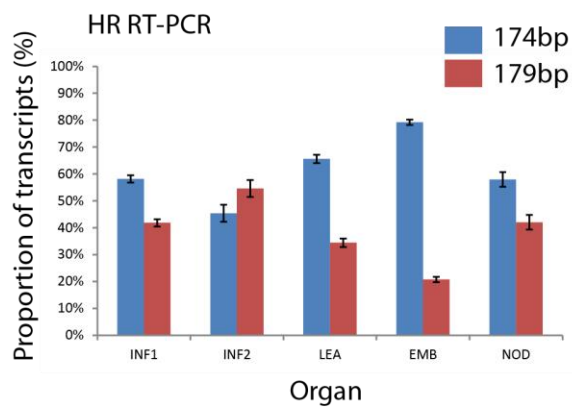
a. 110_Clathrin adaptor complex subunit orthologue

AS1 174bp

AS2 179bp

alt 5'ss

exon 3 — intron 3 — exon 4

b.

c.

100nt   150nt   200nt   300nt

Relative Flourescence Units (RFU)

INF1

INF2

LEA

EMB

NOD

174bp   179bp

d. HR RT-PCR

Proportion of transcripts (%)

174bp
179bp

Organ

e. RNA-seq

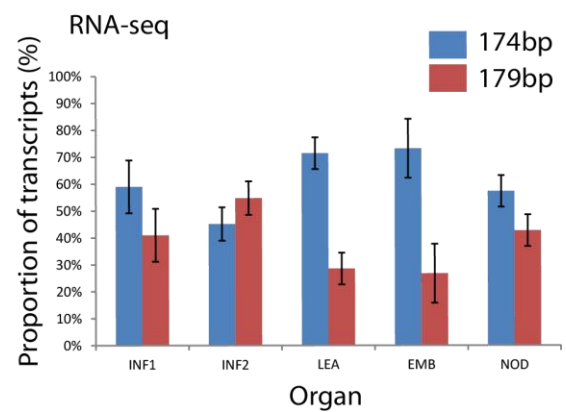Proportion of transcripts (%)

174bp
179bp

Organ

14

Figure 1. High resolution RT-PCR analysis of a single alternative splicing event.

a. Schematic representation of a barley clathrin adapter complex subunit orthologue protein (MLOC_54446). Coding regions are shown as an open blue box, untranslated regions are shown as a black box and the introns are shown as a line. The alternative 5' splice site event is indicated and the products of HR RT-PCR with primer pair 110F and R (arrowed) are shown with expected RT-PCR product sizes. **b**. Tablet visualisation (**26**) showing individual sequence reads covering intron 3 between exon 3 and exon 4. The exon regions are shown in grey while the intron sequence removed is shown in red. Individual reads show alternative selection of an alternative 5' splice site (arrow). **c.** Electropherograms showing representative examples of the output from the ABI 3730 sequencer. The HR RT-PCR products are identified as peaks 174bp and 179 bp (see Fig. 1a). The X-axis indicates length of HR RT-PCR product in base pairs (bp) and the Y-axis indicates the relative fluorescence units. Results are shown for the splicing analysis of five different barley organs: inflorescence (INF1 and INF2); leaf (LEA); embryo (EMB) and internode (NOD) tissues. See text for more detailed information. **d** and **e**. Graphs indicates the proportion of spliced products (174 bp – blue bar and 179 bp – red bar) expressed as a percentage (%) across the barley organs from an HR RT-PCR (**d**) and an RNA-seq experiment (**e**). Error bars represent standard errors between three biological repeats.