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ORIGINAL ARTICLE

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Distribution of actin gene isoforms in the *Arabidopsis* leaf measured in microsamples from intact individual cells

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Abstract The contents of single plant cells can be sampled using glass microcapillaries. By combining such single-cell sampling with reverse transcription-polymerase chain reaction (RT-PCR), transcripts of individual genes can be identified and, in principle, quantified. This provides a valuable technique for the analysis and quantification of the intercellular distribution of gene expression in complex tissues. In a proof-of-principle study, the cellular locations of the transcripts of the eight isoforms of actin (*ACT*) expressed in *Arabidopsis thaliana* (L.) Heynh. were analyzed. Cell sap was extracted from epidermal and mesophyll cells of leaves of 3- to 4-week-old plants. Single-cell (SC)-RT-PCR was used to amplify the actin transcripts using specific primer pairs for *ACT1*, *2*, *3*, *4*, *7*, *8*, *11* and *12*. Only *ACT2* and *ACT8* were found in epidermal and in mesophyll cells. In individual trichomes, in addition to *ACT2* and *ACT8*, *ACT7* and *ACT11* transcripts were detectable. By employing the already well-characterized actin system we demonstrate the practicality and power of SC-RT-PCR as a technique for analyzing gene expression at the ultimate level of resolution, the single cell.

Keywords Actin · *Arabidopsis* (actin) · Gene expression · Pressure probe · Single-cell sampling

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Abbreviations GUS: β-glucuronidase · PCR: polymerase chain reaction · RT: reverse transcription · Rubisco: ribulose-1,5-bisphosphate carboxylase-oxygenase · SC: single cell

Introduction

Plant organs are composed of numbers of distinct cells and cell types, each contributing to the behaviour of the entire structure. In order to understand this division of labour it is essential to have techniques that can quantitatively describe the behaviour of the different cells. Increasing numbers of techniques to study the physical and chemical properties of individual living cells within organs are becoming available (reviewed by Davies 2001). One approach is based on the use of the cell pressure probe (Tomos and Leigh 1999; Tomos et al. 2000). Crucially, the anatomical complexity of plants complicates the analysis of gene expression at a cellular level. Techniques involving the quantification of individual mRNAs within RNA samples extracted from whole tissues [e.g. hybridization, quantitative reverse transcription-polymerase chain reaction (RT-PCR)] are sensitive and simple to carry out, but necessarily represent an average of the various different cell types. Several general approaches have been employed to focus this to cellular and even sub-cellular resolution (Kehr 2001). These include in situ hybridization (McFadden 1994), elegant hand-dissection (Outlaw and Zhang 2001), and advanced laser-capture micro-dissection (Simone et al. 1998). Promoter-reporter expression systems (for reviews, see Guivarch et al. 1996; Hanson and Köhler 2001), although popular, have certain drawbacks. Plants that carry a reporter transgene must be produced, and the number of individual genes that can be studied is limited to the number of transgenic lines that can be produced.

Recent advances in sampling techniques using single plant cells (see Tomos et al. 1994, 2000; Tomos and Leigh 1999; Tomos and Sharrock 2001) offer the

prospects of analyzing levels of individual mRNAs from material isolated from intact fully physiological single cells. A fine microcapillary is employed to extract cell sap from the individual cells, after which RT-PCR is performed (Lambolez et al. 1992; Karrer et al.1995; Brandt et al. 1999; Koroleva et al. 2001). The critical advantage of single-cell (SC)-RT-PCR is that it enables expression to be measured directly from living tissue at the finest spatial resolution possible, that of the individual cell. Moreover, because the microcapillary can also be used to measure, in single cells, parameters such as membrane potential, ionic status, metabolite contents and wall mechanical properties (Lambolez et al. 1992; Tomos and Leigh 1999), changes in these can be linked in turn to gene expression (Koroleva et al. 2001).

To demonstrate the practicality of the microcapillary technique for analyzing gene expression in *Arabidopsis*, we have applied SC-RT-PCR to determine the expression of different actin gene isoforms in different cell types within leaf tissue. The actin gene isoforms are particularly well suited for such a study, as the *Arabidopsis* genome potentially encodes 10 isoforms, of which 8 are expressed at detectable levels (McDowell et al. 1996b; McKinney and Meagher 1998). Information on the distribution of the isoforms is already available following RT-PCR analysis of excised tissues and in vivo expression of a β-glucuronidase (GUS) reporter gene linked to specific actin promoters (An et al. 1996b; Huang et al. 1996, 1997; McDowell et al. 1996a). Recently, Kandasamy et al. (1999) used class-specific antibodies to visualize the distribution of the protein. We have specifically chosen the actin gene family for this study to permit a direct comparison of observations between the SC-RT-PCR and promoter-reporter techniques.

Each actin isoform is likely to be present at low abundance (in tomato, actin accounts for approximately 0.04% of the poly(A)$^+$mRNA; Karrer et al. 1995). In contrast, the small subunit (*RBCS*) of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) is present in high abundance in the mesophyll cells – for which it can act as a practical cell-type marker gene.

Here, we describe the use of SC-RT-PCR to determine the distribution of expression of all eight actin isoforms between the epidermis, mesophyll and trichomes of leaves of *Arabidopsis*. Our results confirm and extend those of Meagher and co-workers (Meagher and Williamson 1994; An et al. 1996a, b; McDowell et al. 1996b; Meagher et al. 1999), and demonstrate the power of the SC-RT-PCR technique in the analysis of the differential expression of multiple genes within a single homologous family.

Materials and methods

Plant growth

Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Col-0 were surface-sterilized, dried and plated on 0.8% agar containing half-strength Murashige-Skoog (Sigma) medium with added 20 mM sucrose. The seeds were incubated at 4 °C in the dark for 4 days before being moved to a growth cabinet under continuous light (70 μmol photons m^{-2} s^{-1}) and 20 °C until the first true leaves were emerging (7–10 days). At this point, seedlings were transferred to compost (pre-treated with the insecticide Intercept, supplied by Ropax, Lancs., UK) and grown at 21 °C with either a 14-h photoperiod (55 μmol photons m^{-2} s^{-1}), or under continuous light. Samples were taken from the upper surface of rosette leaves of plants 3–4 weeks after the onset of illumination, by which time the sampled leaf blades (not including petiole) were 10–15 mm long.

Extraction of sap from individual cells

Using a binocular microscope and a silanized glass microcapillary previously sterilized at 200 °C, samples of sap were isolated from single cells, or from groups of adjacent cells of the same type, from rosette leaves of intact plants (Tomos et al. 1994; Brandt et al. 1999). The turgor pressure of each cell was sufficient to drive a proportion of its contents into the capillary upon puncture. Cell sap was extracted directly from individual epidermal cells. In the case of the mesophyll, however, the capillary had first to pass through the overlying epidermis. Individual trichomes were cut off under a dissecting microscope (using a scalpel).

Extraction of poly(A)$^+$mRNA

Poly(A)$^+$mRNA was purified from bulk leaf samples using magnetic oligo(dT)$_{25}$-coupled Dynabeads (Dynal AS, Oslo, Norway). Approximately 4 mg of leaf tissue was ground in 100 μl of lysis/binding buffer and centrifuged for 5 min at 14,000 *g*. The supernatant was added to 20 μl of pre-washed Dynabeads suspended in lysis/binding buffer. After 5 min incubation at room temperature with periodic gentle agitation, the beads were washed twice with Buffer A and twice with Buffer B (Dynal). The beads, with bound poly(A)$^+$mRNA, were re-suspended in 50 μl of 10 mM Tris (pH 7.5) at 4 °C, the tube was placed on a magnetic rack (Dynal) and the supernatant was discarded immediately prior to adding the reverse transcriptase mixture. Individual excised trichomes were ground in 50 μl of lysis binding buffer and were then processed by adding to 20 μl of beads, as above.

Poly(A)$^+$mRNA was purified from single-cell sap samples using Dynabeads according to the same procedure. Within seconds of being extracted from the cell into the capillary, the single-cell saps were expelled into a 1.5-ml Eppendorf tube containing pre-washed beads suspended in 20 μl of lysis/binding buffer. When several cells were sampled the procedure depended on the cell type. The groups of epidermal samples were all sequentially collected in the capillary before ejection. Mesophyll samples take longer to locate and were ejected singly into the lysis/binding buffer. Subsequent incubation (10–15 min), washing and re-suspension were as above, except that only one wash was performed with Buffers A and B.

RT-PCR analysis

Beads with hybridized poly(A)$^+$mRNA were resuspended in 20 μl cDNA synthesis reaction mixture containing 1 μl reverse transcriptase (Sensiscript; Qiagen, Dorking, UK), 2 μl of each dNTP (each at 5 mM), 2 μl 10× RT-buffer (Qiagen), 1 μl of 40 U/μl RNAsin (Promega, Southampton, UK) and 14 μl of water. The reaction was carried out for 1 h at 37 °C followed by cooling on ice and storage at 4 °C prior to PCR. Each cDNA sample was then divided into three aliquots to permit three PCR reactions (two actin-specific primer pairs and the *RBCS* control) to be performed.

Primer pairs specific for each actin isoform were used. For *ACT1*, *2*, *3*, *7* and *8*, primers were as described by McKinney and Meagher (1998) except that the restriction-site sequences were omitted. Novel primers were designed for *ACT4*, *11* and *12*, the reverse primers being complementary to the 3′ untranslated region. Primers specific to the small subunit of Rubisco (*RBCS*) were also used. The primers and the expected sizes of the PCR products are shown in Table 1.

Table 1 The PCR primer sequences used in this study. Primers used for amplification of *ACT1*, *2*, *3*, *7* and *8* were as described by McKinney and Meagher (1998) except that they were synthesized without the restriction-site sequence. New PCR primers were designed for *ACT4*, *11* and *12* and *RBCS*. The predicted fragment sizes for amplification products of processed mRNA and from genomic DNA are indicated

	Forward primer (5′-3′)	Reverse primer (5′-3′)	Approximate PCR fragment size (bp)
Actin 1	CTGAGTTCAAAGTGATCATTTTTCA	TTACATAATAATTGAAAAATTGAAG	250
Actin 2	CTAAGCTCTCAAGATCAAAGGCTTA	ACTAAAACGCAAAACGAAAGCGGTT	200
Actin 3	TTGAGCTTGAAGTTAAGTCTGCTTC	TATCAAATAACCTCTCTAAACTTGG	250
Actin 4	TCCGGTGTCCGGAAGTTCTGTTC	CCGTCTTCGTTTGGTGATCTTAGG	400 (cDNA) 500 (genomic DNA)
Actin 7	CTAAGTGTGTCTTGTCTTATCTGGT	TGAACCAAGGACCAAATATAATATG	270
Actin 8	CTAAACTAAAGAGACATCGTTTCCA	GTTTTTATCCGAGTTTGAAGAGGCT	250
Actin 11	AGTACAGTGTCTGGATTGGAGGC	CAACAAAGTCGATGAACAAAAGGC	200 (cDNA) 286 (genomic DNA)
Actin 12	TCCGGTGTCCTGAAGTTCTGTT	GCAAATGGCTTCAGGGTGAA	200 (cDNA) 500 (genomic DNA)
RBCS	CTATGGTCGCTCCTTTCAACGG	TGCAACCGAACAAGGGAAGC	386 (cDNA) 584 (genomic DNA)

Six microliters of cDNA-synthesis reaction mixture was used as the template for PCR in reactions containing 15 µl of Hotstar *Taq* DNA polymerase master mix (Qiagen), 10 pmol of each specific primer and 1.5–3 mM Mg^{2+} final concentration, in a total reaction volume of 30 µl. Reactions were performed in a thermo-cycler (Techne, Cambridge, UK) as follows: 15 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 59 °C and 60 s at 72 °C. The series was terminated with 5 min at 72 °C.

PCR products were separated by electrophoresis in 2% agarose-TBE-ethidium bromide gels. RT-PCR products from bulk leaf samples were extracted from the gel using a Geneclean kit (Qbiogene, Harefield, UK) according to the manufacturer's instructions and sequenced by MWG Biotechnology (Milton Keynes, UK).

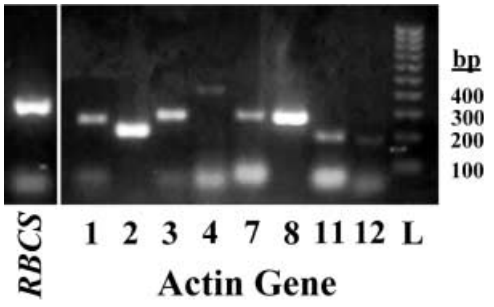

Fig. 1 Separation by agarose gel electrophoresis of RT-PCR products of actin transcripts in whole leaves of *Arabidopsis thaliana*. mRNA was isolated from approximately 4 mg of whole-leaf material and used as template for RT-PCR. Amplification products were visualized by ethidium bromide staining. The DNA size ladder (*L*) is shown on the right. The sizes (in base pairs) for the four smallest markers are given. *RBCS* Small subunit of Rubisco

Results

Of the 10 actin gene family members, 8 had been previously reported to be expressed in *Arabidopsis* leaves (McDowell et al. 1996b). We confirmed this by RT-PCR analysis of poly(A)$^+$mRNA extracted from 4-mg samples of young rosette leaves of 3- to 4-week-old plants (Fig. 1). All eight actin and the *RBCS* PCR products were of the predicted size. Controls, in which mRNA was omitted, did not produce any PCR products (not shown). We subsequently confirmed the identity of the PCR product for each actin isoform and *RBCS* by sequencing. The primers for *ACT4*, *ACT11*, and *RBCS*, were designed to span intron sequences in the genomic sequence. We always observed single bands of the size corresponding to the spliced mature transcript (Fig. 1). The absence of any bands of the size predicted from the genomic DNA sequence (see Table 1) demonstrates that contamination by genomic DNA is absent.

Careful manipulation of the microcapillary generally yielded epidermal cell sap uncontaminated by other cell types. However, before puncturing an underlying mesophyll cell, the capillary had first to be passed through the overlying epidermal cell. Thus, the possibility of contamination of mesophyll mRNA samples by epidermal cell mRNA could not be excluded. The sap of trichomes was too viscous to sample conveniently with the microcapillary; therefore, individual trichomes were obtained by micro-excision.

In individual mesophyll cells, only *ACT2* and *ACT8* were detected (Fig. 2A). Using *ACT2* primers, we obtained a PCR product of the appropriate size in 9 out of 10 samples. A product using *RBCS* primers was always observed; *ACT8* was slightly more variable. In plants grown under continuous light, *ACT8*, was present in 8 out of 10 samples, but in only 1 out of 4 samples from plants grown under a 14-h photoperiod. Similar results were obtained for *ACT2* and *ACT8* when samples were pooled from groups of three mesophyll cell samples in order to increase the likelihood of detecting rare messages. Repeating the analyses at least three times, we did not detect PCR products for *ACT1*, *3*, *4*, *7* or *12* in either single or three-pooled mesophyll cell samples under either growth condition. *ACT11* was detected once in a pooled sample from three mesophyll cells.

Samples from individual and three-pooled cells of the upper epidermis were obtained and analyzed for each of the eight actin isoforms. A few such epidermal samples

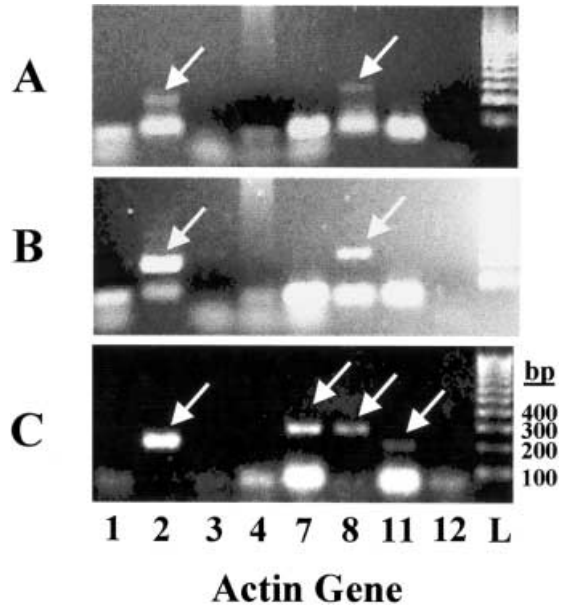

Fig. 2A–C Separation by agarose gel electrophoresis of RT-PCR products of actin transcripts in single cells of *Arabidopsis* leaves. Amplification products were visualized by ethidium bromide staining. **A** Sap from single mesophyll cells; **B** sap from single epidermal cells; **C** individual trichomes. The DNA size ladder (*L*) is shown on the right along with the sizes (in base pairs) of the four smallest markers. The same DNA ladder was used in **A–C**. PCR products of the expected size are indicated by *arrows*

were positive for *RBCS*, indicative of mesophyll cell contamination; the remaining samples were negative. Using the actin primers, only the PCR products for *ACT2* and *ACT8* were routinely observed in samples from single cells (Fig. 2B), *ACT2* in 16 out of 20 samples and *ACT8* in 12 out of 20. For both isoforms, no *RBCS* amplification was observed in at least three of the samples in which the appropriate actin mRNA was also detected. As in the case of the mesophyll cells, most of the samples positive for *ACT8* were obtained from plants grown under continuous light conditions. Only one out of five was positive in plants grown in a 14-h photoperiod. Even with pooled samples from three epidermal cells, we were still unable to amplify *ACT1*, *3*, *4*, *7*, *11* or *12*, although the mRNA present in these samples was presumably undegraded because we were able to detect *ACT2* routinely.

Although we could see no cellular material attached to the excised trichome, we routinely observed the *RBCS* PCR product, indicative of mesophyll contamination. Four actin species, *ACT2*, *7*, *8*, and *11*, were amplified (Fig. 2C). Since we did not have a control specific for epidermal cell contents, we cannot be sure that the presence of *ACT2* and *8* is not the result of contamination by epidermal cells. However, because *ACT7* and *11* were detected neither in single-cell epidermal samples nor in mesophyll samples, we conclude that expression of these two species is specific to trichomes. We did not detect *ACT1* and *3* in trichomes, in contrast to the results of An et al. (1996a).

Discussion

This work extends that of Karrer et al. (1995); Brandt et al. (1999) and Koroleva et al. (2001) in establishing SC-RT-PCR as a technique applicable to plant cells. Here we have demonstrated that the technique can be applied successfully to identify patterns of expression of the different but related members of a single family of homologous genes, each of which is present at only moderate abundance (Karrer et al. 1995). In mesophyll cells we always obtained a PCR product for Rubisco and for *ACT2* in 9 out of 10 samples, suggesting that the RNA was substantially intact. We have as yet no explicit estimation of the detection limit, although in every case it was much better than that required to measure *ACT2* expression. In other systems, e.g. tomato, actin is considered to be a moderately abundant message, comprising 0.04% of the total poly(A)$^+$ mRNA (Karrer et al. 1995). Since *ACT2* mRNA is likely to comprise the majority of actin mRNA species in leaf tissue, this implies that the detection limit is better than 100 molecules per cell.

When analyzing patterns of gene expression in single cells, the possibility of contamination by the contents of cells of a different type remains present. Here we used the Rubisco transcript as a marker for mesophyll contamination. The *RBCS* transcript was undetectable in the majority of our epidermal cell samples, although some samples did give a positive signal. This apparent expression of *RBCS* in a proportion of epidermal cells is most likely a result of contamination. In barley, several supposedly mesophyll transcripts are found in epidermal samples on an all-or-none basis (our unpublished data). Our working hypothesis is that these transcripts are indeed limited to the mesophyll cells.

Because Rubisco is the most abundant transcript in green plants, comprising up to 4% of the total mRNA (Thompson et al. 1983), other contaminating mesophyll transcripts, if any, should be present at a considerably lower level (in the case of most transcripts, several orders of magnitude lower). In a technique like single-cell sampling, the possibility of contamination of the sample can never be entirely eliminated. However, the use of cell-type-specific markers (such as Rubisco) is likely to constitute an essential and discriminatory control in SC-RT-PCR studies, since any contaminated samples can be easily identified and rejected.

In the previously published work, more isoforms were claimed for each cell type than reported here (see Table 2). Why might this be? Reporter-gene studies measure the activity of a protein that is not naturally a constituent of plant cells, unlike SC-RT-PCR, which directly quantifies mRNA in *non-genetically modified* plants. The GUS protein is extremely long-lived; moreover, the turnover rates of the GUS and actin transcripts are unlikely to be identical. Consequently GUS activity constitutes only an indirect measure of gene activity. Patterns of organ and tissue expression derived from

Table 2 A comparison summary of the distribution of GUS staining found in leaves of actin promoter-GUS transgenic plants by Meagher and colleagues, and the results obtained in this work

Tissue type	GUS expression (Meagher and co-workers)[a]	SC-RT-PCR (this work)
Epidermis	*ACT2*, *ACT8* (An et al. 1996b) *ACT11* (Huang et al. 1997) *ACT7* (McDowell et al. 1996a)	*ACT2*, *ACT8*
Mesophyll	(cell type not specified)	*ACT2*, *ACT8*
Trichome	*ACT2*, *ACT8* (An et al. 1996b) *ACT11* (Huang et al. 1997) *ACT7* (McDowell et al. 1996a) *ACT1*, *ACT3* (An et al. 1996a) RT-PCR analysis	*ACT2*, *ACT8* *ACT11* *ACT7* RT-PCR analysis
Bulk leaves	*ACT2*, *ACT8* (An et al. 1996b) *ACT11* (Huang et al. 1997) *ACT7* (McDowell et al. 1996a) *ACT1*, *ACT3* (An et al. 1996a) *ACT4*, *ACT12* (Huang et al. 1996)	*ACT2*, *ACT8* *ACT11* *ACT7* *ACT1*, *ACT3* *ACT4*, *ACT12*

direct measurements of mRNA abundance may differ from those based on actin promoter-GUS constructs (An et al. 1996a, b; McDowell et al. 1996a) because GUS protein may still be active long after levels of actin mRNAs have fallen below the detection limit.

As in mesophyll and epidermal cells, both *ACT2* and *8* mRNAs were present in trichomes or trichome-associated cells, which additionally expressed *ACT7* and *11*. No traces of *ACT1*, *3*, *4* and *12* were found in any sample. The consistent presence of *RBCS* mRNA in trichomes was surprising since contamination by mesophyll cells seems unlikely. Indeed we cannot exclude the possibility that *RBCS* is expressed in the trichomes themselves.

The ability to amplify *ACT2* or *RBCS* transcripts in each sample is a control for the absence of mRNA degradation, crucial where evidence for no or low expression of a message is sought. Because the abundance of all the actin mRNAs combined is approximately 100 times lower than that of *RBCS* mRNA (Karrer et al. 1995), *ACT2*, which appears to be expressed in all cell types, will provide an important internal control for mRNA integrity in future studies.

The apparent variation in *ACT8* expression between plants grown under different light conditions provides some evidence that the differential expression patterns of *ACT8* may be under environmental control. Expression of *ACT1*, *3*, *4*, and *12* was never detected in either epidermal or mesophyll samples. These isoforms have been previously reported to be present mainly in reproductive organs (An et al. 1996a; Huang et al. 1996). We did detect these transcripts in excised leaf samples, presumably from the vascular tissue.

We did not detect the *ACT7* transcript in epidermal or mesophyll samples. McDowell et al. (1996a) reported GUS activity as "blue patches" in young leaves, the staining tending to diffuse and disappear in older leaves. It is possible, but unlikely, that all our epidermal and mesophyll samples taken were from "white" patches. Alternatively leaves may already have been of an age at which only the extremely stable GUS protein remained, whilst the actin mRNA had been degraded below the detection limit. This might also be the case for *ACT11*, for which Huang et al. (1997) reported GUS activity in young leaves.

SC-RT-PCR is a powerful tool with which to analyze gene expression at the resolution of the cell. Here we demonstrate the applicability of the technique. Naturally occurring transcripts (in this case *RBCS*) can be used as both positive and negative controls. This can be further extended using tissue-specific transgenes (Brandt et al. 1999). Here we have used a simple PCR detection system to determine whether the level of particular individual transcripts is greater than an as yet not fully quantified detection limit. However, the technique should be fully adaptable to authentically quantitative PCR techniques, and we propose to continue this work in this direction. We further plan to take advantage of micro-array technologies to analyze simultaneously a much larger number of transcript species.

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