

Profiling Gene Expression in Germinating *Brassica* Roots

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Abstract Based on previously developed solid-phase gene extraction (SPGE) we examined the mRNA profile in primary roots of *Brassica rapa* seedlings for highly expressed genes like *ACT7* (actin7), *TUB* (tubulin1), *UBQ* (ubiquitin), and low expressed *GLK* (glucokinase) during the first day post-germination. The assessment was based on the mRNA load of the SPGE probe of about 2.1 ng. The number of copies of the investigated genes changed spatially along the length of primary roots. The expression level of all genes differed significantly at each sample position. Among the examined genes *ACT7* expression was most even along the root. *UBQ* was highest at the tip and root–shoot junction (RS). *TUB* and *GLK* showed a basipetal gradient. The temporal expression of *UBQ* was highest in the MZ 9 h after primary root emergence and higher than at any other sample position. Expressions of *GLK* in EZ and RS increased gradually over time. SPGE extraction is the result of oligo-dT and oligo-dA hybridization and the results illustrate that SPGE can be used for gene expression profiling at high spatial and temporal resolution. SPGE needles can be used within two weeks when stored at 4 °C. Our data indicate that gene expression studies that are based on the entire root miss important differences in gene expression that SPGE is able to resolve for example growth adjustments during gravitropism.

Keywords *Brassica* · Gene expression profiling · mRNA quality · Solid-phase gene extraction

Abbreviations

<i>ACT7</i>	<i>Actin7</i>
cDNA	Complementary DNA
DEPC	Diethylpyrocarbonate
<i>GLK</i>	Glucokinase
MZ	Meristematic zone
qPCR	Quantitative PCR
RIN	RNA integrity number
RT	Room temperature
rRNA	Ribosomal RNA
SPGE	Solid-phase gene extraction
<i>TUB</i>	<i>Arabidopsis Tubulin1</i>
<i>UBQ</i>	<i>Arabidopsis Ubiquitin1</i>
EZ	Elongation zone
RS	Root–shoot junction

Introduction

Multiple studies have demonstrated that RNA quality influences the analysis of gene expression profiles (Liebhaber 1997; Mitchell and Tollervey 2000; Guhaniyogi and Brewer 2001). Therefore, the extraction of mRNA is a critical initial step in gene expression analysis. RNA quality is typically assessed by computational methods, e.g., the degradometer software (Auer et al. 2003) or the RNA integrity number (RIN, Schroeder et al. 2006). The assessment of RNA quality depends on the presence and magnitude of 28S and/or 18S ribosomal RNA (rRNA) on gels (Sambrook and Russel 2001) or equivalent electrophoretic separations. Contamination and degradation of rRNA is presumed to indicate mRNA degradation despite the fact that mRNA and mRNA fragments are significantly smaller than rRNA.

Diverse methods are used to isolate mRNA. Often cellulose-bound oligo-dT_[15–20] (Aviv and Leder 1972; Chirgwin

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et al. 1979) coated magnetic beads are used to selectively hybridize to the ubiquitous polyadenylated tail end of mRNA from previously extracted total RNA. However, any process to purify mRNA has the potential to degrade mRNA as a result of contaminants or enzymatic activity in the crude sample. The maceration of cells, tissue, or cellular components releases nucleases and other hydrolytic enzymes that can degrade mRNA. In addition to yield and quality of the mRNA, gene expression depends on temporal, spatial, or developmental changes in the sampled material (Choi and Kim 2007; Bengtsson et al. 2005; Toufeer et al. 2011). Therefore, gene expression profiling improves with the enhanced temporal and spatial resolution of sampling. To bypass purification and expedite nucleic acid extraction, we have designed solid-phase gene extraction (SPGE), a process based on solid probes that are surface-coated with oligo-dTs or gene-specific oligonucleotide sequences (Scherp and Hasenstein 2008). SPGE does not require additional purification of the sample and takes less than 2 min from extraction to reverse transcription. The fast turnaround reduced RNA degradation and does not destroy the sampled material (Scherp and Hasenstein 2008).

Previous SPGE studies used borosilicate glass needles that were brittle and difficult to handle. Here, we demonstrate an improved protocol that uses steel needles and an improved coating protocol to investigate gene expression profiling during early root growth in *Brassica* seedlings and show the variability of transcripts of stably expressed genes (housekeeping or reference genes) that are often employed for data normalization in Quantitative PCR (qPCR; Kim et al. 2003; Jain et al. 2006). We selected actin7 (*ACT7*), tubulin1 (*TUB*), and ubiquitin (*UBQ*) to examine changes of expression as a function of time and the developmental state of the tissue. We also investigated glucokinase (*GLK*), a low expressed gene in roots, to demonstrate the suitability of SPGE to investigate the variability of low-expression genes.

Materials and Methods

Plant Material and RNAs Extraction

Seeds of *Brassica rapa* were germinated at 25 °C for 48 h in the dark. Total RNA was extracted from roots and primary root of germinated seeds of *Brassica* using the innuPREP RNA mini kit (Analytik Jena, Germany) and mRNA was purified from total RNA using Oligotex mRNA Mini Kit (Qiagen, USA). All procedures for RNA extraction were according to manufacturer's protocols.

Preparation of SPGE Probes and RNA Extraction

Stainless steel acupuncture needles (#6, SEIRIN, Japan) were submersed into Piranha solution (H₂SO₄:H₂O₂, 3:1 v/v) for

30 min and then rinsed with distilled sterile water (3×250 µl). The rinsed needles were incubated in 1 % aminopropyltrimethoxysilane in 95 % v/v acetone/water for 30 min then washed with 100 % acetone (3×100 µl) and dried for 1 h at 150 °C. The dried needles were incubated in 0.2 % 1,4 phenylene-diisothiocyanate in 10 % (v/v) pyridine/dimethylformamide solution for 2 h and washed in acetone and then methanol (3×5 min in 100 µl each). The needles were incubated in 0, 1, or 5 µM NH₂-oligo (dT)₁₅ nucleotides in 100 mM sodium carbonate buffer (pH 9) at 37 °C for 1 h. The coated needles were rinsed with 1 % ammonium hydroxide (5 min) and distilled sterile water (3×5 min) and then air-dried at RT.

mRNA was extracted by inserting the prepared needles into 20 µl of previously extracted total RNA (2.5 µg/µl) in a PCR tube or by directly inserting the probe into the primary root (about 1.5 mm thick and completely pierced) of *Brassica* seedlings at different locations. After 1 min, the probes were removed and washed with wash buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5) for 1 min. Then quality and quantity of RNA were assessed, reverse-transcribed, and quantified by qPCR. The reusability of SPGE probes was investigated after repeated incubation of needles in 80 °C diethylpyrocarbonate (DEPC)-treated water for 3 min to release mRNA before subsequent extractions.

Assessment of Amount and Quality of RNAs

The mRNA-loaded SPGE probes were incubated for 3 min in 16 µl 80 °C DEPC-treated water to release mRNA. The released mRNA was analyzed using HT RNA Kit with LabChip® Systems (LabChip® 90, Caliper Life Sciences, USA) according to manufacturer's protocol and by formaldehyde gel electrophoresis (1 % agarose, 1× MOPS buffer (20 mM 3-[*N*-morpholino] propane-sulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 6.8–7.0, 2 % formaldehyde) and identical running buffer (60 V for 1.5 h). The ratio between the absorbance at 260 and 280 nm was used to estimate RNA purity and quantity using a NanoDrop ND-1000 spectrophotometer (ND-1000 UV/VIS, NanoDrop Technologies, USA); these data were also used to normalize SPGE extractions. To assess the robustness of SPGE hybridized mRNA, SPGE-extracted mRNA was stored at 4 °C or RT for up to 72 h before being examined by RT-qPCR based on *ACT7* (Table 1).

cDNA Synthesis and qPCR

Complementary DNA (cDNA) was synthesized from total RNA and SPGE-extracted mRNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, USA). Reverse transcription was carried out in a PCR tube containing one SPGE needle, 16 µl DEPC-treated water, and 4 µl reverse transcription mixture (final concentration: 1 mM dNTP, 1× reverse transcription buffer, and 50 U Multiscribe™

Table 1 Primer sequences of *ACT7*, *TUB*, *UBQ*, and *GLK* genes

Gene (accession no.)	Primer	Sequence (5'→3')	T_m (°C)	Amplicon (bp)	Efficiency (%)
<i>ACT7</i> (NM_121018.3)	Forward	AGCTTCGTGTTGCACCTGAA	58	120	104.3
	Reverse	ACATGGCAGGGACATTGAAAG			
<i>TUB</i> (D78496.1)	Forward	CTCGATGGCCTCAACCTTA	58	162	104.7
	Reverse	ATGTTGCTCTCGGCTTCTGT			
<i>UBQ</i> (Z24738.1)	Forward	GGAGAGCAGTGACACCATCGA	58	120	103.8
	Reverse	GCCAAGGTACGACCATCTCA			
<i>GLK</i> (AT3G20040)	Forward	TGATTGCTGCTCGGTTACAGAA	58	102	99.9
	Reverse	CACATTCATCAGCTTGAAGCAG			

reverse transcriptase) at 25 °C for 10 min, then at 37 °C for 2 h, and finally at 85 °C for 5 min to inactivate the reverse transcriptase. The synthesized cDNA was diluted 1:3 in DEPC water and stored at −20 °C until further use. The qPCR reaction mixture [final concentration: 1× Power SYBR® Green PCR Master Mix, 100 nM primers for *ACT7*, *TUB*, *UBQ*, and *GLK* (Table 1), 2 µl diluted cDNA] was adjusted to 10 µl with water. qPCR was performed after 10 min at 95 °C for polymerase activation, and cycling between 95 °C (15 s) and 58 °C (1 min) for 40 cycles in an Applied Biosystems StepOne™ system (Applied Biosystems). QPCR products were subjected to melt curve analysis (60 to 95 °C at 3 °C min^{−1}) and capillary electrophoresis using a QIAxcel system (Qiagen).

External Standards for qPCR

For quantification of copy numbers, cDNA standards for each investigated gene were synthesized from total RNA of 10 mm long root of *Brassica* using high-capacity cDNA reverse transcription kit (Applied Biosystems). Individual PCR products of each investigated gene were amplified with Phusion® high-fidelity PCR kit (New England Biolabs, USA) according to the manufacturer's protocol, using primers shown in Table 1. Individual PCR products were analyzed to confirm the presence of a single product using a QIAxcel system (Qiagen). The product was purified using the QIAquick PCR Purification Kit. Purified PCR products were quantified using a NanoDrop ND-1000 spectrophotometer and serially diluted (1:10–1:100,000). The exact quantity and known sequence of the PCR product allowed calculation of copy numbers per dilution based on quantification cycle (C_q) values of each serial-dilution. The copy numbers N_c for the genes were calculated as $N_c = \{ \text{initial DNA concentration (ng/µl)} \times \text{added volume(s) (µl)} \times 10^{-(\text{the slope} \times C_q - \text{the intercept})} \times (6.022 \times 10^{23}) \} / [\text{amplicon length (bp)} \times 650 \{ \text{average mass of a base pair} \} \times 10^9 \text{ (conversion to ng)}]$.

Statistical Analysis

Analysis of variance (ANOVA) was performed with Statistix 7.0 (Statistix, USA) based on three independent biological replicates and three technical replicates.

Results

Quality of the Extracted RNA

The absorbance ratio (260/280 nm) of total RNA extracted directly from roots of *Brassica* and mRNAs isolated from total root RNA (2.5 µg/µl RNA) were 2.0 and 2.1, respectively. Labchip electropherograms of DEPC-treated water showed only a marker peak (Fig. 1a). The quantification of 28S and 18S (rRNA) on agarose gels and Labchip electropherograms showed that the total RNA was of good quality (Fig. 1b). mRNA comprised 0.97 % of total RNA. Primary rRNA (5 s, 18 s, and 28 s) peaks were not found on electropherogram of mRNA (Fig. 1c) or of mRNA extracted by SPGE (Fig. 1d). The largest fragments from total RNA, bulk-purified mRNA, and SPGE-extracted mRNA were 7, 100, 6,200, and 4,400 nt, respectively (Fig. 1b, c, and d).

Extraction Capacity of SPGE Probes

We coated probes with 1 or 5 µM NH₂-oligo (dT)₁₅ nucleotides and used noncoated probes as control. These probes (0, 1 and 5 µM NH₂-oligo (dT)₁₅-coated) extracted on average 1.5, 9.2, and 9.6 ng mRNA, respectively. The uncoated surface most likely binds mRNA nonspecifically; the coating intensity was saturated at 1 µM (Fig. 2). When SPGE needles were incubated for 1 min in wash buffer at RT, the mRNA amount associated with the needle decreased by 20 % compared with the nonwashed needles, but *ACT7* quantity did not change (Fig. 3). The amount of mRNA extracted directly from the root tissue by SPGE was 2.1±0.2 ng per needle compared with 9.1±1.0 ng obtained from total *Brassica* RNA extract. This difference is likely related to the lower density of mRNA in tissue samples as compared to the solubilized mRNA.

SPGE Probe Stability and Storage

After washing and incubation in 80 °C DEPC-treated water for 3 min, each needle released up to 9.1 ng mRNA. However, repeated extractions with the same probes resulted in a drastic decrease of *ACT7* quantification (Fig. 4).

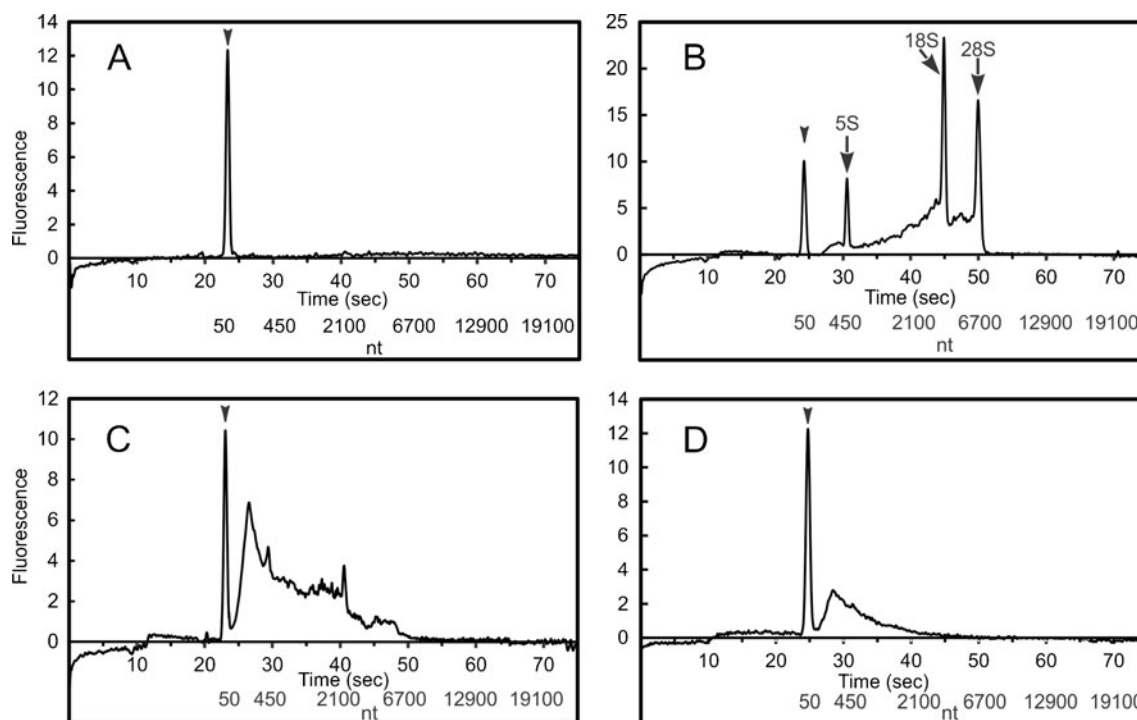


Fig. 1 LabChip® 90 electropherograms of DEPC-treated water (**a**), total RNA extracted from roots of *Brassica* seedlings (**b**), messenger RNA (mRNA) extracted from total RNA (**c**), and mRNA extracted from total

RNA by SPGE (**d**). Peaks represent the lead marker (50 nt, arrow head) or ribosomal RNAs

The amount of mRNA and number of copies of *ACT7* extracted after storage of SPGE probes at 4 °C or RT was not affected. However, extended storage at RT before extraction gradually reduced the measured *ACT7* quantity over time (Fig. 5a). In contrast, probes stored at 4 °C retained their extraction capacity for 2 weeks (Fig. 5a). mRNA that was extracted and stored on SPGE probes was not detectably degraded within 3 days based on the extracted quantity of *ACT7* (Fig. 5b).

Spatial and Temporal Profiling of Gene Expression in Roots

The number of copies of highly expressed genes (*ACT7*, *UBQ*; Fig. 6a) at five positions in 10-mm-long primary roots of

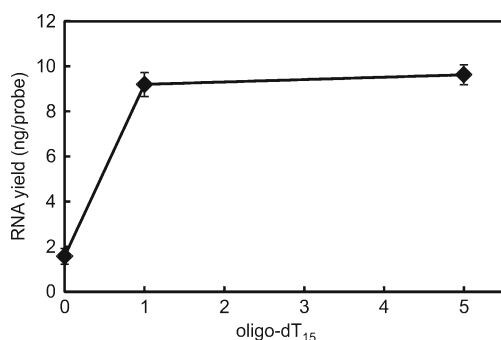


Fig. 2 Amounts of SPGE-extracted mRNA with probes coated with different concentrations of the NH₂-oligo (dT)₁₅ nucleotides; average ± SE, *n*=9. Least significance difference for SPGE-extracted mRNA is 0.41 ng (*p*<0.05)

Brassica varied with the sampling position. *UBQ* in the root-shoot junction (RS; Fig. 6a, sample position 5) showed the highest copy number, about 4,800. Low expressed genes (*GLK*, *Tub*, Fig. 6b) had the lowest copy number in the meristem zone (sample position 1). The highest copy number for *ACT7*, *TUB*, and *GLK* was found at sample positions 2 and 3, respectively (Fig. 6). This data set illustrates that the expression of all examined gene differed significantly along the root.

We used two genes of different expression levels (*UBQ*, high expression and *GLK*, low expression), to investigate the dynamic range and profiling of genes over time at three positions (MZ, EZ, and RS; Fig. 7). The expression of these two genes showed clearly varied during the first 24 h after primary root emergence. SPGE covers a dynamic range of eightfold at the EZ after 24 h for *UBQ*. The dynamic range increased to 107-fold at RS after 24 h for *UBQ* compared to that of *GLK*. Similar to positional changes, the expression of all examined genes changed over time. The copy number of *UBQ* fluctuated but remained generally low in the MZ and EZ but expression at the RS region strongly increased after 18 h. In contrast, the expression of *GLK* showed a steady increase at all sampling times for RS and EZ but remained low in the MZ (Fig. 7). Therefore, the expression patterns of the examined genes and most likely other, if not most, genes vary over time and position. We verified that the differences in gene expressions are not the result of different extraction yields based on tissue or developmental stage. The yield per probe at different extraction sites was not different and averaged 2.1±0.2 ng (data not shown).

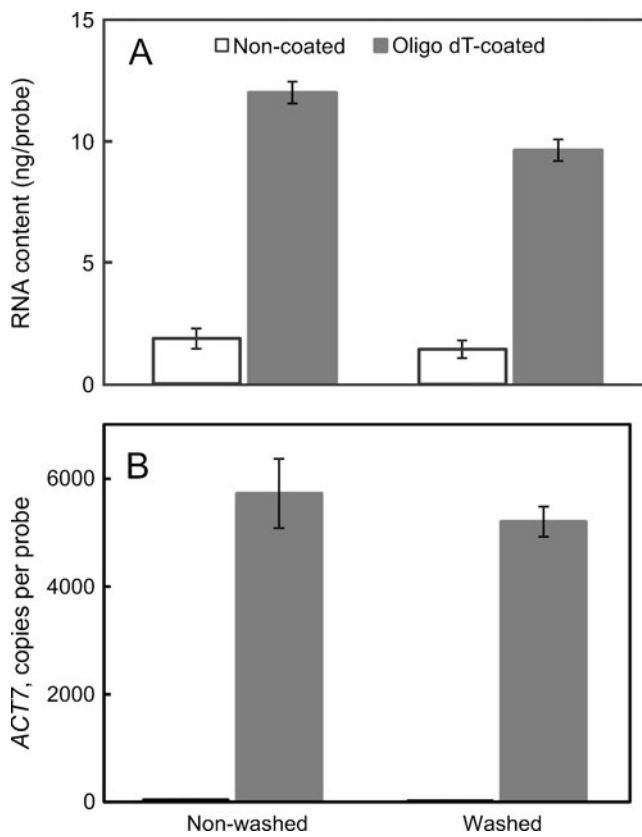


Fig. 3 SPGE-extracted mRNA (a) and relative amount of *ACT7* per SPGE probe (b). SPGE probes were either incubated in total RNA of *Brassica* root and directly reverse transcribed or washed with buffer before reverse transcription and quantification; average \pm SE, $n=9$. Least significant difference ($p<0.05$) for SPGE-extracted mRNA is 0.40 ng and for *ACT7* copies is 1,164 copies

Discussion

Quality assessment of RNA samples is of central importance for the evaluation of gene expression profiles (Mueller et al. 2004; Li and Reilly 2008). Commonly used gel electrophoresis

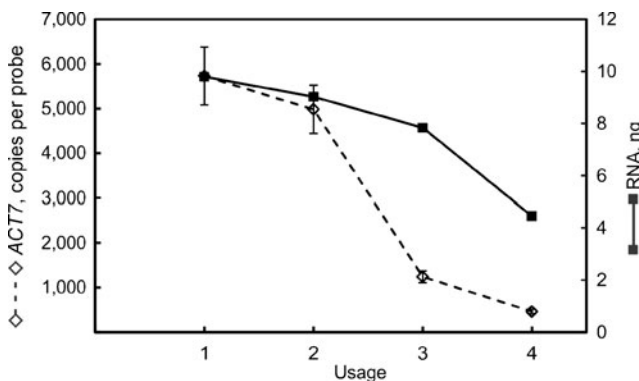


Fig. 4 Quantity of mRNA extracted from total RNA by SPGE (—) and number of copies of *ACT7* after multiple usage of SPGE probes (- - -). The probes were washed before release of mRNA (80 °C for 3 min). Average \pm SE, $n=9$. Least significance difference ($p<0.05$) for amounts of SPGE-extracted mRNA is 0.39 ng, and for number of copies of *ACT7* is 1,403 copies

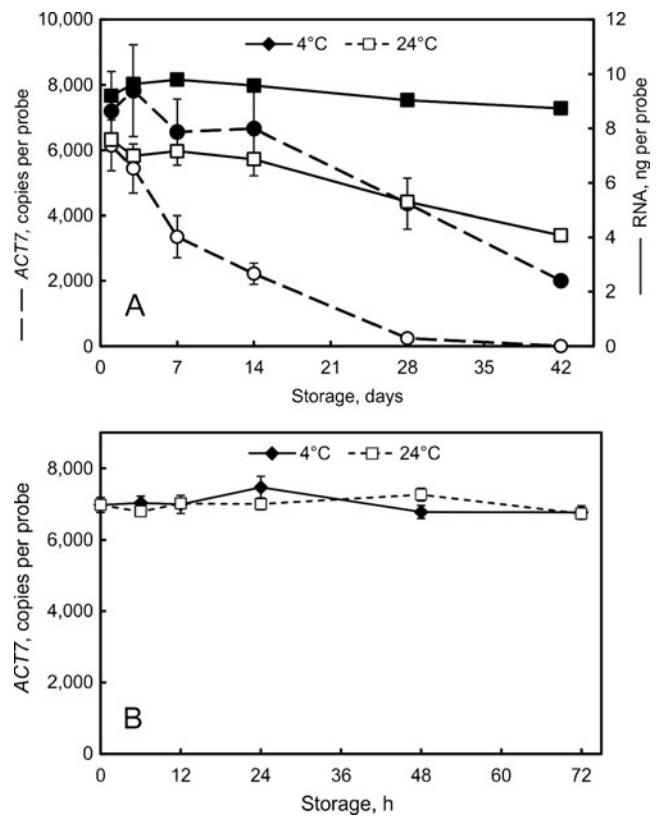


Fig. 5 Amount of mRNA (squares) and copies of *ACT7* per probe (circles) as a function of storage at 4 °C (filled symbols) or RT (open symbols) after storing SPGE probes prior to extraction (a) and the stability of mRNA on the SPGE probe prior to reverse transcription (b). Average \pm SE, $n=9$. The least significance difference ($p<0.05$) for SPGE-extracted mRNA is 0.35 ng and for *ACT7* copy number is 1046 (Fig. 5a). The least significance difference for SPGE-extracted mRNA is 208 copies (Fig. 5b)

consumes a large amount of RNA and provides limited information on RNA quality. Recent developments of high-sensitivity systems, such as Agilent's Bioanalyzer or Caliper's LabChip® technology, provides detailed information about RNA quality from capillary electropherograms (Kuschel 2000; Vitale 2001a, b). However, these systems assess total RNA that includes components, such as tRNA or rRNA, that do not contribute to reverse transcription or qPCR but are used to determine the quality of the extraction. In contrast to generic extraction of RNA, SPGE-extracted mRNA only contains mRNA (Fig. 1). The tailing shape of the electropherogram (Fig. 1d) indicates that mRNAs were not contaminated by other RNAs and that different size classes (lengths) of mRNAs are present. Although a “RIN” number cannot be calculated from this sample, our data show that SPGE-extracted material is suitable for gene expression profiling.

mRNAs sizes vary widely according to the size of the encoded polypeptides. However, several studies reported that over 90 % of the cDNAs synthesized from mRNA were <4.0 kb (Smith et al. 2001; Zhu et al. 2001). Based on these general observations and our results, which correspond to the <4 kb size

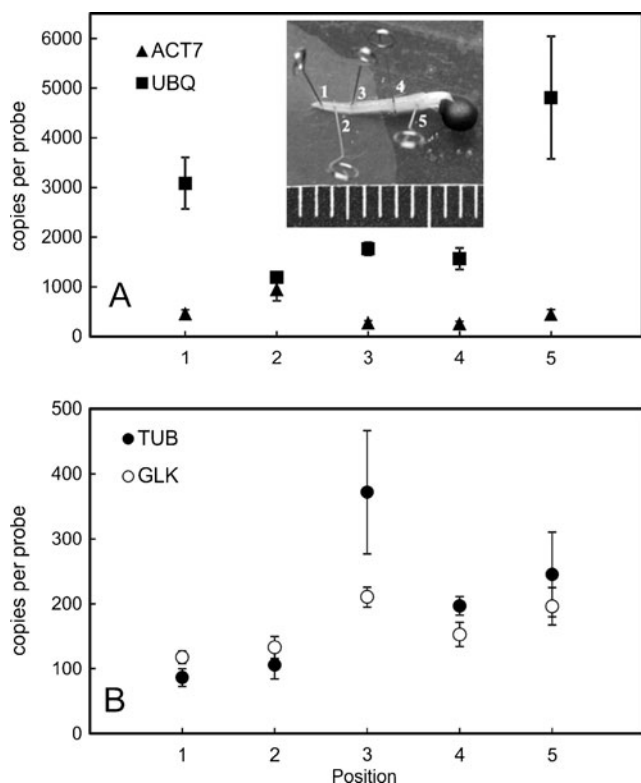


Fig. 6 Relative expression of highly expressed genes (*ACT7*, *UBQ*; **a**) or low expressed genes (*TUB*, *GLK*; **b**) along 10-mm-long primary *Brassica* roots. Root were sampled about 36 h after germination at five positions (**a**, insert) and the copy number of *ACT7*, *UBQ*, *TUB*, and *GLK* determined at each sample position [1 (*tip*) to 5 (*root–shoot junction*); average \pm SE, $n=9$]. Least significance differences at $p<0.05$ for copy numbers of *ACT7*, *UBQ*, *TUB*, and *GLK* are 61, 229, 32, and 21 copies, respectively

range (Fig. 1), SPGE extraction is suitable for gene expression profiling.

Oligo-(dT)₁₅ concentration of 1 μ M was optimal for oligo-dT coating regardless of the linking procedure (Scherp and Hasenstein 2008). Good hybridization of poly-A tail with oligo-dTs is indicated by unchanged number of copies of *ACT7* after washing (Fig. 3). However, the loss of RNA by washing suggests that not all RNA is stably hybridized to the oligo-dT-coated probe. On the other hand, the extracted mRNA did not degrade during dry storage up to 72 h (Fig. 5b). Future experiments will show the optimal length of the oligo-dT sequence. The higher stability of hybridization with longer oligo-dT sequences has to be weighed against increased entanglement that can lead to less specific hybridization and may affect coating efficiency.

Regardless of these details, the data show that RNA purification after SPGE extraction is unnecessary. The elimination of purification steps, the reusability of the probes, and extended storability by simple refrigeration make SPGE a valuable and convenient mRNA extraction technique. The lack of DNA contamination can also be assessed based on the uniform amplification of *UBQ*. The primers were selected

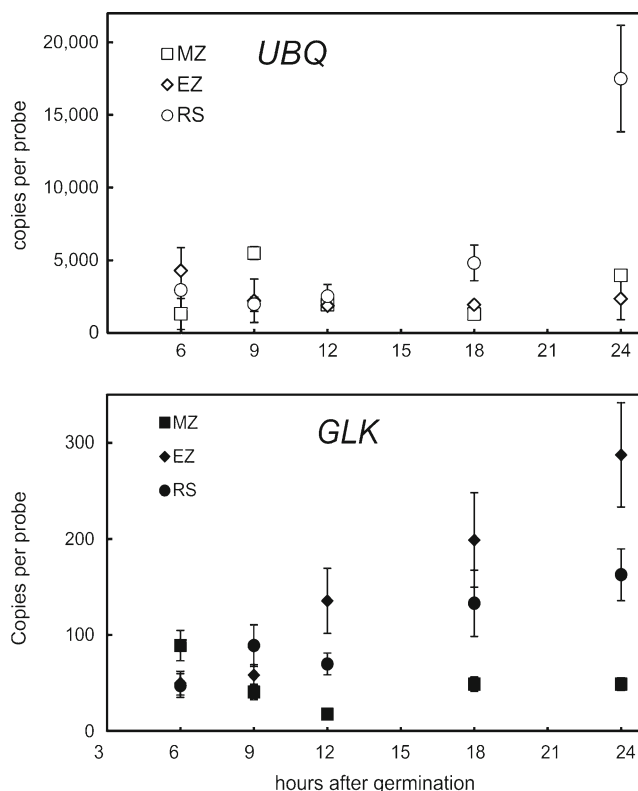


Fig. 7 Expression of ubiquitin (*UBQ*, top) and glucokinase (*GLK*, bottom) of *Brassica* primary roots. The roots were sampled at the meristem (*square*), elongation zone (*diamond*) and root-shoot junction (*circle*) between 6 and 24 h after germination; average \pm SE, $n=9$. Least significance differences ($p<0.05$) for *UBQ* copy numbers in MZ, EZ, and RS are 164, 683, and 666 copies, respectively; for *GLK* in MZ, EZ, and RS, the values are 5.7, 18.7, and 10.8 copies, respectively

to span an 80 bp intron such that DNA contamination would have produced amplicons of 200 bp. However, only the 120 bp amplicon was detected and the melt profile was uniform.

The total RNA-derived mRNA amount represents the needle capacity; the tissue-derived value is reduced probably because of less than saturating quantities of RNA in the sampled cells and nonuniform distribution of RNA. For example, the volume occupied by cell wall, vacuoles, or intracellular spaces contain less or no RNA compared with cytoplasm. In contrast, a RNA-containing solution provides a uniform and enriched concentration of mRNA with which the probe coating can hybridize.

Gene expression changes are highly dynamic, and gene activation patterns vary and often are complex (Rogina et al. 1998; Maizel and Weigel 2004). Expression of genes also depends on tissues and developmental stages (Montoliu et al. 1989) and formation of organs is based on successive gene expression during development (Gilliland et al. 2003). Therefore, profiling of spatio-temporal gene expression is critical for the understanding of the interaction of genes within specific tissues or sites of an organism during development.

Considering the genes that we examined in this study, it is important to correlate gene expression patterns with established physiological changes. Actin is involved in the control of diverse processes including synthesis of the cell plate and correcting cell-division plate alignment, cell shape determination, establishment of polarity, cytoplasmic streaming, organelle movement, and tip growth (Staiger and Schliwa 1987; Staiger and Lloyd 1991; Meagher and Williamson 1994). The assessment of actin is further complicated by multiple isoforms, which represent a large gene family (Meagher and McLean 1990; Meagher 1991). *ACT7* encodes one of the six distinct vegetative actin genes (Gilliland et al. 2003). In meristematic and elongation zones of roots, cells exhibit dramatic rearrangements of the actin cytoskeleton (Baluška et al. 1997, 2001; McDowell et al. 1996) and *ACT7* is the most strongly expressed isoform in young seedling of *Arabidopsis* (Gilliland et al. 2003; Basu et al. 2003) in line with our finding that the number of copies of *ACT7* in 2-day-old *Brassica* roots is highest in the EZ (Fig. 6a). The strong expression of actin makes it a favored reference gene that has been widely used and examined (Khanlou and Van Bockstaele 2012; Lopez-Pardo et al. 2013; Migocka and Papierniak 2011; Xiao et al. 2012); however, these papers also illustrate the intrinsic uncertainty related to any type of reference gene. Our data indicate that *ACT7* is not uniformly or constantly expressed; thus, a systematic assessment of gene expression depicts transcription activity better than referencing a single gene.

UBQ is another frequently used reference gene (Khanlou and Van Bockstaele 2012; Migocka and Papierniak 2011; Xiao et al. 2012) and reported to be stably expressed in the root of *Salvia miltiorrhiza*. Its 1,000-fold higher expression compared to *TUB* (Yang et al. 2010) is in line with the high expression seen in our data (Fig. 6) but was not examined for temporal and spatial variability as in this study. The upregulation of *UBQ* in RS (position # 5) preceded the development of root hairs. *UBQ* is linked to root and lateral root development (Nodzon et al. 2004; Nishizawa et al. 2008; Prasad et al. 2010; von Schaeuwen et al. 1990) and changes related to these processes question the suitability of this gene as reference gene. Our results showed upregulation of *ACT7* and *TUB* in EZ (positions # 2 and 3), indicating that elongation growth of roots correlates with the expression of cytoskeleton proteins.

As a common metabolic resource, carbohydrates influence plant growth and development such as internode elongation, root formation, and leaf development (Dickinson et al. 1991; Weber et al. 1998; Jang and Sheen 1994). The copy number of *GLK*, one of the genes related to sugar sensing and signaling, was low but variable (Fig. 6b). Because *GLK* gradually increased in EZ and RS, root development and maturation relies on *GLK* activity; in contrast, the constant expression in MZ suggests that mitotic growth is less sensitive to carbohydrate feedback than expansion growth, possibly as a result of increased cell wall biosynthesis in elongating cells.

We show that the expression of each gene depends on the position in the root. This result is important as for most genetic analyses, tissues are pooled, and the size, age, and developmental stage of different roots may confound the results. Although it is possible that a combination of genes (e.g., *ACT* and *UBQ*) and/or pooled tissues may result in more stable expression, such combinations reduce the sensitivity of gene expression studies. In contrast, SPGE-based high-resolution time-course profile allows a detailed assessment of the expression profiles for several genes in roots. The number of genes is only limited by the reverse transcription yield.

In summary, our results illustrate the delicate task of identifying stable reference processes genes or stages during early stages of seedling growth; the results corroborate SPGE as suitable tool for gene expression profiling at high spatial and temporal resolution with a large dynamic range. Essential information for multiple genes is crucial to understanding regulatory networks during development and is provided by this methodology.

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