Over the past few years high-throughput platforms for real-time quantitative PCR have become widely available. The cost of RNA extraction from a large number of samples are, however, quite notable. One method that stands out with respect to free up- or downscaling of sample size and reliability is the isolation of mRNA using oligodeoxythymidylate [oligo(dT)$_{25}$]-coated magnetic particles. In combining this magnetic separation of mRNA with real-time reverse transcription PCR (RT-PCR), we have achieved a highly reproducible, economic, and fast way of analyzing large sample numbers. One difficulty that has so far prevented the fusion of these techniques relates to accurate mRNA quantification. We present a solution to this problem that enables excellent adjustment of cDNA amounts prior to the real-time PCR. Furthermore, as the mRNA is rapidly isolated from crude plant extracts, our method is widely applicable to herbaceous plant species and various tissue types without cumbersome adjustments. Although designed and tested here for plants, we anticipate that the principles should be applicable to gene expression studies in any other organism. Lastly, due to its flexibility, the method presented here can easily be adapted to specific requirements of various users and has great potential for further automation.

INTRODUCTION

Transcriptional profiling has become a standard technique in the analysis of gene expression with conventional quantification methods such as Northern blot analyses, RNase protection assays, and competitive reverse transcription PCR (RT-PCR) being increasingly replaced by large-scale approaches. High-density DNA oligonucleotide or cDNA arrays are now available for a range of organisms and species, allowing for gene expression studies at the whole genome scale. Microarray technology is mostly used to compare RNA populations from limited sample numbers (1,2) so that the extraction of the template is not a limiting factor. High-fidelity cDNA amplification protocols (3–5) allow for comparison of small amounts of tissues and thereby information on tissue specificity. A complementary approach is the expression analysis of a limited number of genes of interest on large sample sets using real-time quantitative RT-PCR (6,7). Such sample sets may arise from sampling different genotypes (e.g., transgenic lines or ecotypes), differently treated source material (e.g., different growth conditions), or from serial sampling of different tissue types at several developmental stages. In these cases, numerous RNA extractions and reverse transcription reactions have to be performed. With the high-throughput capacity of quantitative PCR platforms, these initial steps are quickly becoming a real bottleneck for large scale expression analyses.

A key factor with respect to RNA extraction is the quality of the isolated mRNA (8,9). We developed a high-throughput technique for gene expression profiling from small amounts of plant tissues using the combination of mRNA extraction by oligodeoxythymidylate [oligo(dT)$_{25}$]-coated magnetic beads and quantitative RT-PCR, with beadbound cDNA as template. While the high quality, robustness, and sensitivity of mRNA isolations via magnetic beads (as well as their ability to isolate mRNA from a wide range of tissues and even single cells) has long been known (10–13), this technique has so far not been routinely used in quantitative RT-PCRs (14,15). One reason for this could be the difficulty to determine the cDNA concentration of the resulting solid-phase cDNA library, which prevents a reliable adjustment of cDNA concentrations among samples prior to the real-time PCR run. In this report we offer a solution to this problem of even sample loading, which makes magnetic quantitative RT-PCR applicable to a broad range of biological samples and adaptable to a wide range of platforms and scales of investigation.

MATERIALS AND METHODS

Plant Material

Arabidopsis, wheat, and canola plants were grown in controlled growth chambers under 10, 13, and 12 h photo-period, respectively, irradiance of 130, 350, and 600 µE m$^{-2}$ s$^{-1}$, day and night temperatures of 21°/19°, 23°/21°, and 26°/22°C, and 65%–75% relative humidity.

Rice plants were grown in a glasshouse at 25°C (day) and 22°C (night) and 65%–75% relative humidity under a 14-h light period (natural day length extended whenever necessary by supplemental lighting of approximately 300 µE m$^{-2}$ s$^{-1}$).

Buffers and Solutions

For lysis buffer: 100 mM Tris, pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM dithiothreitol (DTT); for wash buffer 1: 10 mM Tris, pH 8.0, 150 mM LiCl, 1 mM EDTA, 0.1% LiDS; for wash buffer 2: 10 mM Tris, pH 8.0, 150 mM LiCl, 1 mM EDTA; for 5x RT buffer: 250 mM Tris, pH 8.3, 250 mM KCl, 50 mM MgCl$_2$, 0.5 mM DTT; for elution buffer: 2 mM EDTA, pH 8.0. All solutions were prepared with diethylpyrocarbonate (DEPC)-treated Milli-Q® water (Millipore, North Rye, New South Wales, Australia).

RNA Extraction and First-Strand Synthesis

Plant material was ground to a fine powder under liquid nitrogen and stored at -80°C until use. All washing/elution...
steps were performed by relocation and magnetic separation on a 96-well magnetic platform (Dynal MPC®-9600; Invitrogen, Mount Waverley, Victoria, Australia). Prior to use, 10 µL magnetic bead solution [Dynabeads® Oligo(dT)25; Invitrogen] were aliquoted to each well and washed twice with 200 µL lysis buffer. Thirty milligrams of tissue powder were incubated in 300 µL lysis buffer for 10 min at 1800 rpm and room temperature on an orbital shaker. Extracts were centrifuged twice at 10,500 × g and 15°C for 10 min, and 230 µL of the final supernatant were transferred to the washed magnetic beads. After a 10-min mRNA annealing phase to the oligo(dT)25 primer at room temperature and subsequent magnetic separation, the beads were washed twice in 200 µL wash buffer 1, once in wash buffer 2, twice in ice-cold 1× RT buffer, and finally resuspended in 40 µL DEPC-treated Milli-Q water. After incubation at 70°C for 5 min and chilling on ice, first-strand cDNA synthesis was carried out by adding 10 µL RT master mix containing 4 µL 5x RT buffer, 0.25 µL 100 mM DTT, 1 µL 100 mM dNTPs, 40 U RNasin® RNase Inhibitor, 50 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (all from Promega, Hawthorn, Victoria, Australia), and 4.25 µL DEPC-treated Milli-Q water and incubating for 1 h at 42°C. After two washes with 200 µL 1x RT buffer, the mRNA was eluted from the bead-bound cDNA at a melting temperature of 95°C for 10 min. The concentration of mRNA in the chilled supernatant was determined using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Beads were washed with 100 µL Milli-Q water, the cDNA concentration adjusted to 2 ng/µL with sterile Milli-Q water and stored at -80°C. For the subsequent quantitative PCR, samples were diluted 1:10 in Milli-Q water, unless stated otherwise.

For comparison, RNA extraction and first-strand cDNA synthesis were also performed from 60 mg ground rice leaf tissue using the RNAqueous® kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. Two micrograms of total RNA were then transcribed into first-strand cDNA as described previously. Ten nanograms of first-strand cDNA were used in quantitative PCR.

Real-Time PCR

Quantitative PCR and cycle threshold (Ct) value determination were carried out using an ABI PRISM® 7700 or 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and accompanying software according to the manufacturer’s instruction. Reactions for the ABI PRISM 7700 contained 5 µL bead-bound cDNA (1 ng mRNA equivalent), 5 µL primer mix (1.2 µM each), and 10 µL 2x SYBR® Green PCR Master mix (Applied Biosystems). When using the ABI PRISM 7900HT system, the reaction volume was halved to 10 µL. Primer sequences are listed in Table 1. After each run, a melting curve analysis was performed to verify target specific product amplification. Determination of PCR efficiencies for individual reactions was performed as described in Ramakers et al. (16).

RESULTS AND DISCUSSION

Binding Capacity of Magnetic Beads and Comparison with Total RNA Extraction Methods

In order to calibrate the method, we used RNA yields typically obtained
with conventional total RNA isolation methods (in our hands 30–60 µg total RNA/100 mg fresh tissue, depending on tissue type). Assuming that mRNAs constitute about 1% of the total RNA pool (17), and knowing that magnetic beads have a binding capacity for mRNA of 10 ng per microgram, we inferred that 50 µg of beads (i.e., a 3- to 5-fold excess) should be more than sufficient to bind all the mRNA extracted from 30 mg of any given plant tissue, thus retaining absolute transcript amounts. With these assumptions we conducted quantitative RT-PCR experiments comparing C\textsubscript{t} values obtained from kit extracted total RNA versus bead isolated mRNA from the same tissue samples. The data in Figure 1 show that there was no significant difference in OsAPT1 transcript levels determined by the two methods (Student’s t-test, \( p = 0.05 \)). This is all the more remarkable as the amounts of cDNA used for quantitative PCR were determined quite differently. In the case of the column-based extraction, 2 µg total RNA were used in the reverse transcription reaction, then 10 ng total RNA equivalents were used for the real-time PCR run. In the case of the magnetic bead extraction, mRNA was eluted from the first cDNA strand after the RT reaction, the mRNA concentration was determined, and then 0.1 ng mRNA equivalents from the solid phase cDNA library was used for real-time PCR. A typical mRNA amount obtained in a given sample was 90 ng from 30 mg of plant tissue.

**Handling of Magnetic Beads in Real-Time PCR-Dilution Series**

A primary concern was the suitability of a solid phase bead-bound cDNA library for real-time PCR. In order to address that issue and test for any problem in keeping the beads in suspension (e.g., electrostatic charging or formation of magnetic bead agglomerates), we performed quantitative PCR on cDNA dilution series. Figure 2 shows that AtAPT1 expression could readily be detected over five orders of magnitude, giving the same calculation for the logarithmic regression of 0.999 in both cases. This indicates that the solid phase cDNA stays in suspension for long enough to allow for dilution steps, and that the transcribed cDNA was of high quality, suitable to amplify AtAPT1 in as little as 0.0005 ng total cDNA. These results also show that the magnetic beads do not cause quenching of the fluorescence signal and that their autofluorescence is negligible (data not shown). As a further test that our magnetic quantitative PCR method produces comparable expression data to conventional methods, we prepared cDNA from mRNA eluted from the magnetic beads prior to the reverse transcription step shown in Figure 1A (right panel). The C\textsubscript{t} values obtained from this cDNA reaction in liquid phase were similar to those from the solid phase bead-bound cDNA in the same volume reaction (fold expression difference of 1.4 ± 0.8 across three genes tested using 0.05 ng rice cDNA).

**Reproducibility of Procedure**

As a first test of the reliability of our magnetic quantitative PCR method, we prepared first-strand cDNA from three independent Arabidopsis tissue harvests. Under our assumptions that the amount of mRNA eluted from the beads after...
the reverse transcription step provides a good measure of the amount of final product (i.e., first-strand cDNA), and that the transcript levels of our chosen control genes (AtAPT1, AtPDF2, AtUBQ10, At2g32170, and AtUBC10) are good measures for the amount of cDNA loaded (18), there should be little variation among Ct values for control gene expression in different biological replicates. This was indeed the case (Figure 3A). Reproducibility across all five control genes and between replicates proved to be very high, with expression levels for any one control gene varying by <1.7-fold. In addition we tested cDNA from 40 individual rice or Arabidopsis plants (Figure 3B). Again we found little variation in the adenine phosphoribosyltransferase 1 (APT1) expression level for rice with a Ct(OsAPT1) of 16.9 ± 0.7 (mean ± SD). In Arabidopsis, there was more sample-to-sample variation [Ct(AtAPT1) of 22.7 ± 2.6], reflecting a greater variation in leaf age and growth conditions at the time of sampling compared with the rice material (see Figure 3).

mRNA Isolation from Different Plant Species and Tissues

One important shortfall of many column-based RNA isolation kits is that they are less suitable to obtain high-quality RNA from plant tissues that are rich in polysaccharides, lipids, or polyphenol compounds, such as seeds or inflorescences (19–21). The magnetic beads could be better suited to these tissues, as they immediately withdraw the mRNA from the inhibitory environment (Figure 1A). To test this notion, we isolated mRNA from a range of tissues, including seeds, and from several species (Arabidopsis, canola, and wheat). Exactly the same procedure was used for all samples (see Materials and Methods section). Figure 4 shows that all tissues from the three plant species yielded mRNA that was readily transcribed into cDNA, as judged from the characteristics of the quantitative PCR amplification plots, with Ct values for the APT1 control gene varying from 23 to 25. Exceptions were wheat and canola roots, with a Ct value of 28 and 29, respectively, as well as Arabidopsis dry seeds and mature siliques, which gave higher Ct values of 34, even with a 10-fold increase in the amount of cDNA used compared with other tissues. At this stage, we cannot conclude with certainty whether this reflects a lower expression of the purine salvage pathway gene in these reproductive and storage tissues or whether cDNA synthesis itself was impaired. Gene expression data from Schmid et al. (22), however, give support to the former hypothesis showing an approximately 16-fold decrease in APT1 transcript levels in dry seeds compared with leaves. It is remarkable that our chosen APT1 control gene was relatively stably expressed across leaf tissues of all species. A notable exception was rice leaf blades, where APT1 was 60-fold more expressed than in leaves of the three other species (Figure 3B).

To our knowledge, this is the first report of a high-throughput RNA isolation procedure able to efficiently deal with such a variety of plant tissues without protocol modifications. The time required from the isolation of mRNA out of the crude extract to the reverse transcription reaction is <1 h, therefore reducing the risk of mRNA degradation. As no centrifugation steps

Figure 4. Quantitative PCR performance for mRNA isolated from a range of tissues in different plant species. cDNA was prepared from different wheat, canola, and Arabidopsis tissues. Half a nanogram of cDNA was used in each reaction, unless indicated otherwise. Amplification curves are expressed as ROX™ passive reference dye-normalized signal intensity, plotted against PCR cycle number. The threshold value used to determine cycle threshold (Ct) values is indicated as a dashed line in each amplification plot. TaAPT1 primers were used for wheat, and AtAPT1 primers were used for Arabidopsis and canola tissues (see Table 1).

Table 1. Specifications of Oligonucleotide Pairs Used in Real-Time Quantitative RT-PCR

<table>
<thead>
<tr>
<th>Annotation</th>
<th>Accession No.</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>PCR Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAPT1</td>
<td>At1g27450</td>
<td>5′-TTGCGAGGTGGTGAAGCTAGAGGT-3′</td>
<td>5′-TGACCAATAAGCCACTAATAG-3′</td>
<td>64</td>
</tr>
<tr>
<td>AtPDF2</td>
<td>At1g3320</td>
<td>5′-TACGGTGCCCAAATAGATGC-3′</td>
<td>5′-GTTCCCAACAGCCTTTGTG-3′</td>
<td>61</td>
</tr>
<tr>
<td>AtUBQ10</td>
<td>At4g5320</td>
<td>5′-CAGCTTCACATTGCTTGCGT-3′</td>
<td>5′-TGACCTCCCGGAGACTCTTCA-3′</td>
<td>71</td>
</tr>
<tr>
<td>At2g32170</td>
<td>At2g32170</td>
<td>5′-GGAGTGTGAGATAATTTGAGCACTTCTGTATAT-3′</td>
<td>5′-GTCTCCCTCTCTCGATACAACCACA-3′</td>
<td>135</td>
</tr>
<tr>
<td>AtUBC9</td>
<td>At4g27960</td>
<td>5′-CAGCTTCACATTTCATGCTATCGTAC-3′</td>
<td>5′-GTAATGTCAGATCTCAGCGACCAA-3′</td>
<td>91</td>
</tr>
<tr>
<td>OsAPT1</td>
<td>Os12g39860</td>
<td>5′-TTGTTGTAAGGAGCCATGTAGAT-3′</td>
<td>5′-ATTCGGCTAGTCTGCTTCATAC-3′</td>
<td>103</td>
</tr>
<tr>
<td>TaAPT1</td>
<td>U22442</td>
<td>5′-TGAGACGCTACGAGGATCCCTCA-3′</td>
<td>5′-GGCCCTCAGACTGTCAGAAGCTC-3′</td>
<td>143</td>
</tr>
</tbody>
</table>

All primer pairs were designed to span exon-exon borders and for a PCR annealing temperature of 60°C. RT-PCR, reverse transcription PCR.

*Primer pairs as in Czechowski et al. (18) with slight modifications to meet the previously mentioned criteria.
are necessary, frequently observed coprecipitation of carbohydrates, phenolic compounds, or cell wall debris from plant samples is circumvented. Furthermore, with the purification being affinity-based, cDNA contamination by genomic DNA is avoided, and no DNaseI treatment is necessary (data not shown). M-MLV reverse transcriptase ensures high-cDNA synthetic activity and has weaker RNase H activity than, for example, Avian Myeloblastosis Virus (AMV) reverse transcriptase, with its lower sequence transcription accuracy being negligible for its application in real-time PCR (23). Of course, small adjustments such as the addition of polyvinylpyrrolidone (PVP) in the lysis step prior to the incubation with the magnetic beads could improve the efficiency of mRNA extraction from problematic tissues (i.e., mature seeds) if required. It should be noted, however, that this method is not suitable to capture mRNA species that do not contain a poly(A) tail or viral RNA molecules.

In conclusion, the extensive testing of reproducibility of cDNA synthesis and real-time PCR performance we present here shows that our method is reliable and sensitive and performs equally well as conventional methods. Its benefits include easy scaling of sample size and amounts of magnetic beads used, low cost, no contamination with genomic DNA and therefore the potential for high-throughput applications. In our lab, high-quality cDNA could be prepared by one person from 48 individual samples within 5 h, at about half the cost of column-based RNA extraction kits. This includes preparation of crude extracts, mRNA isolation, cDNA synthesis, and adjustment of cDNA concentrations for quantitative PCR. Processing time could even be decreased further in a slightly more costly approach where the mRNA would be eluted prior to the reverse transcription step for use in a liquid-phase reverse transcription reaction with free oligo(dT) or similar primers. This would remove the need for washing steps and make pipetting easier. Furthermore, an automated version of this protocol in a 96-well plate format will enable express expression of an even larger number of samples from mRNA extraction to quantitative RT-PCR in <2 days.

One unique feature of our method is that the cDNA concentration is adjusted from the measured mRNA amount eluted from the first cDNA strand after the reverse transcription reaction. This concept is quite different from using total RNA mass for normalization purposes, as the quality of the RNA and the related efficiency of the enzymatic reaction are taken into account. The use of the total mRNA pool as reference point might open up new possibilities for normalization, especially in cases where tissues with significant imbalances between tRNA and mRNA content are analyzed (24). In addition, as our method results in a solid-phase cDNA library, the samples are easily storables and reusable.

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COMPETING INTERESTS

The authors declare no competing interests.

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Address correspondence to Ricarda Jost, Environmental Biology Group, Research School of Biological Sciences, The Australian National University, GPO Box 475, Canberra ACT 0200, Australia. e-mail: ricarda.jost@anu.edu.au

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