



# High Throughput Gene Expression Analysis of *Arabidopsis thaliana* using Quantitative RT-PCR

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## Abstract

Quantitative RT-PCR is a powerful tool for analysis of differential gene expression. Although this technology has been widely used for RNA quantification of individual genes, its application for expression profiling has not been thoroughly explored. DNA Microarrays have been the technology of choice for high-throughput gene expression studies. The power of the DNA microarray lies in its ability to conduct genome-wide expression profiling on hundreds or thousands of genes, making it an ideal method for primary screening of genes differentially expressed in different samples. However, due to poor quantitative resolution, microarray analysis is not suitable for research projects that require accurate RNA quantitation against a large number of samples. The availability of organism-specific primer libraries has eliminated bottlenecks associated with the use of quantitative RT-PCR to make this a cost-effective tool for gene expression analysis. To meet the high-throughput requirements for differential gene expression analysis, an automated method has been developed for quantitative RT-PCR setup using the Sciclone ALH 3000 Liquid Handling Workstation. Reactions were set up using the SYBR<sup>®</sup> Green Quantitative RT-PCR Kit and a Primer Library for *Arabidopsis* Pathogen-Inducible Genes from Sigma-Aldrich. For these studies, total RNA was isolated from pathogen-infected *Arabidopsis* leaves. Data shown here demonstrates the potential of quantitative-RT-PCR for expression profiling of a defined set of genes against a large number of samples.

## Materials

Unless otherwise indicated, all reagents and materials used in this work were obtained from Sigma-Aldrich. Plant samples were obtained through a donation from the Donald Danforth Plant Science Center in St. Louis. GAPDH and 18S primer sets were designed and manufactured using Sigma-Genosys' Custom Oligo Program. For gene expression studies, the Primary Library for *Arabidopsis* Pathogen-Inducible Genes (PRO100) was used. Total RNA was isolated and purified using the GenElute<sup>™</sup> Total RNA Purification Kit (RTN10). The SYBR<sup>®</sup> Green Quantitative RT-PCR Kit (QR0100) was used for all PCR reaction setup.

## Methods

### Human RNA preparation

HeLa cells were grown in Dulbecco's Modified Eagle's Medium, 10% Fetal Bovine Serum, 4 mM L-glutamine, and 1 mM Sodium Pyruvate to about 90% confluency. The medium was discarded and 3 ml of Lysis solution containing 1% 2-Mercaptoethanol was added to lyse the cells and inactivate RNase. After the cells were harvested, the lysed cells were filtered and purified using the GenElute<sup>™</sup> Total RNA Purification Kit.

### Quantitative RT-PCR Reaction Setup

Quantitative RT-PCR reaction setup was performed using an automated method developed for the Sciclone ALH 3000 Liquid Handling Workstation. A master mix was prepared containing 0.2  $\mu$ M of each forward and reverse GAPDH primer. Initial concentration of total RNA was 50 ng/ $\mu$ l and diluted 10-fold for a working stock in subsequent wells. 18  $\mu$ l of master mix was added to each well of a 96-well plate followed by a 2  $\mu$ l addition of RNA.

### Plant Pathogen Treatment

Leaves of a 5-week-old plant were syringe infiltrated with control or treated with *Pseudomonas syringae* pv *tomato* strain DC3000 expressing *avrRpm1*. At timed intervals, punches were obtained from control and treated leaves. Plant treatment was performed at the Donald Danforth Plant Science Center in St. Louis.

### RNA Purification

Total RNA was isolated from the *Arabidopsis* tissues per instructions for the GenElute<sup>™</sup> Total RNA Purification Kit. Genomic DNA was removed by on-column DNase I digestion (Sigma-Aldrich product code: NA6000). Total RNA was eluted in 1x TE buffer and stored at -80 °C until further use.

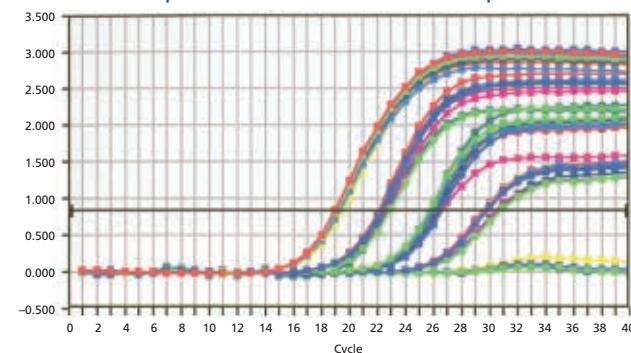
### Gene Expression Analysis

Quantitative RT-PCR reaction setup was performed using the automated method developed for the Sciclone ALH 3000 Liquid Handling Workstation. 200  $\mu$ l of 1x Tris-EDTA Buffer was added to the 96-well plate containing the Primer Library for *Arabidopsis* Pathogen-Inducible Genes. An additional 2-fold dilution of the primer library was performed in another 96-well plate. PCR reactions were set up in two 96-well plates with a total reaction volume of 20  $\mu$ l. A master mix was prepared for each reaction plate, one master mix included RNA from control treated leaves and the second included RNA isolated from disease treated leaves. Quantitative RT-PCR was performed on the MJ Research DNA Engine Opticon2 thermal cycler. After an initial 30-minute reverse transcription step, a total of 40 cycles were run with quantification being measured at 80 °C in each cycle.

Well	Gene Locus Name	Amplicon Length (bp)	Well	Gene Locus Name	Amplicon Length (bp)	Well	Gene Locus Name	Amplicon Length (bp)
A1	At2g32680	210	C9	At1g03220	245	F5	At2g40000	177
A2	At3g56710	150	C10	At1g21250	179	F6	At5g20230	210
A3	At4g02380	150	C11	At5g26920	203	F7	At2g02930	164
A4	At5g60900	164	C12	At4g17500	156	F8	At4g38540	189
A5	At1g18890	242	D1	At5g47200	249	F9	At2g45680	217
A6	At3g16530	156	D2	At4g17490	175	F10	At2g40140	159
A7	At5g44420	152	D3	At3g50770	179	F11	At4g39890	190
A8	At4g04490	158	D4	At3g26820	199	F12	At4g26070	226
A9	At4g11830	151	D5	At4g23150	175	G1	At2g13790	225
A10	At2g44790	186	D6	At1g65970	225	G2	At2g32210	169
A11	At1g10140	213	D7	At4g24340	199	G3	At4g11370	213
A12	At4g12720	160	D8	At1g28480	197	G4	At4g34390	189
B1	At3g04720	228	D9	At1g73800	151	G5	At5g47910	207
B2	At5g18470	223	D10	At2g47130	175	G6	At4g39670	174
B3	At2g28210	184	D11	At4g37520	228	G7	At5g59820	170
B4	At2g31880	159	D12	At2g35980	163	G8	At2g35660	223
B5	At3g59700	157	E1	At3g57260	221	G9	At2g38470	250
B6	At4g16650	229	E2	At3g57240	200	G10	At4g11280	228
B7	At5g60900	194	E3	At2g14610	166	G11	At3g28210	152
B8	At4g23180	238	E4	At4g11890	177	G12	At4g37260	163
B9	At5g45110	175	E5	At4g23130	176	H1	At3g52430	172
B10	At2g14560	150	E6	At4g16260	240	H2	At2g39400	169
B11	At4g36950	195	E7	At3g16530	156	H3	At5g33340	266
B12	At4g14400	161	E8	At3g46090	216	H4	At5g33350	361
C1	At3g56400	216	E9	At4g03450	197	H5	At1g67980	217
C2	At2g31180	236	E10	At1g13470	245	H6	At3g25882	216
C3	At4g25390	182	E11	At5g10760	247	H7	At2g45220	192
C4	At1g47510	179	E12	At5g27600	171	H8	At1g05100	164
C5	At2g22270	196	F1	At1g02920	213	H9	At1g66700	245
C6	At3g04640	217	F2	At2g14560	151	H10	CBP20(At5g44200)	409
C7	At2g32200	161	F3	At4g04490	160	H11	Actin2(At3g18780)	257
C8	At1g05430	166	F4	At1g24140	193	H12	UBC(At5g25760)	217

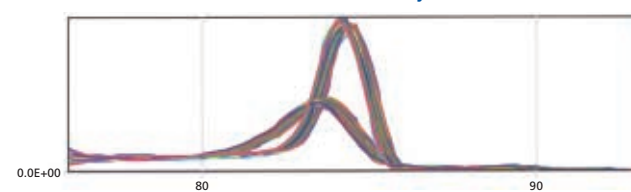
**Table 1. *Arabidopsis* Pathogen-Inducible Genes Primer Library.** This primer library contains 96 sets of gene-specific primers. Ninety-three of the genes are pathogen-inducible genes. The other three genes are housekeeping genes. The housekeeping genes were selected based on their expression patterns. Located at plate positions H10, H11 and H12, the three genes selected were Actin-2, CBP20 and Ubiquitin, respectively.

### Amplification Plot of Human Total RNA Samples



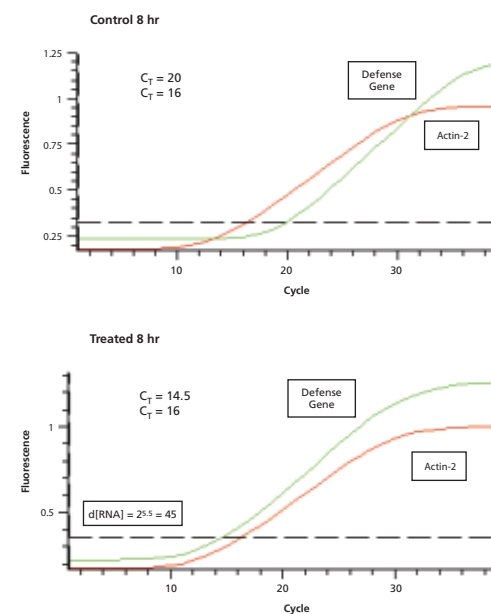
**Figure 1: Amplification Plot of Human Total RNA Dilutions.** Quantitative RT-PCR reaction setup was performed with total human RNA as described in Methods. Amplification was carried out for the following concentrations of total human RNA: 50 ng/ $\mu$ l, 5 ng/ $\mu$ l, 0.5 ng/ $\mu$ l, 0.05 ng/ $\mu$ l, and no template control. The curves on the amplification plot correspond to the concentration of RNA from left to right.

### Cross Contamination Analysis



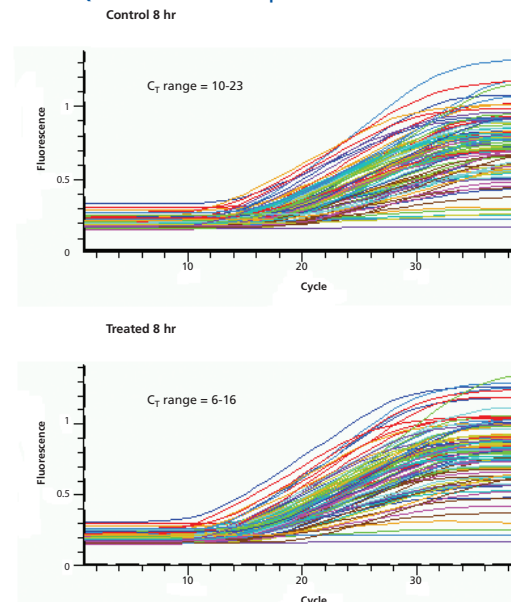
**Figure 2: Melt Curve Analysis of Human Total RNA.** To test for cross-contamination a method was developed in which two different master mixes were dispersed across a 96-well plate to alternating wells. The first master mix contained the GAPDH primer set, and the second mix included the 18S primer set. All samples were subjected to amplification and melt curve analysis on the ABI PRISM<sup>®</sup> 7700 Sequence Detection System. The two primer sets have a differing  $T_m$ , which is indicated in the melting curve. The  $T_m$  of the GAPDH primer set is 84 °C and the 18S primer set had a  $T_m$  of 83 °C.

## Quantitative RT-PCR of Defense-Response Gene Expression



**Figure 3: Real-time Quantification of a Defense-response Gene.** RNA was isolated from untreated and pathogen treated *Arabidopsis* leaves and subjected to Quantitative RT-PCR with 96 different primer sets as described in Methods. The amplification plots shown here represent a single defense gene selected randomly from both the treated and untreated samples. The defense level of the gene was highly induced in pathogen-treated samples (about a 45-fold increase compared to the untreated sample), whereas the internal control actin-2 gene had the same expression level (same  $C_T$  value) in both samples.

## Quantitative RT-PCR Amplification of all 96 Genes



**Figure 4: Real-time RT-PCR Amplification of All 96 Genes from Pathogen-treated and Untreated *Arabidopsis* Leaves.** RNA was isolated from untreated and pathogen-treated *Arabidopsis* leaves and subjected to Quantitative RT-PCR with 96 different primer sets as described in Methods. Many genes were induced in pathogen-treated samples as indicated by the reduced  $C_T$  values. The  $C_T$  for pathogen-treated samples ranged from 6-16, whereas the untreated control ranged from 10-23.

Well	C-8 hour	T-8 hour	Well	C-8 hour	T-8 hour	Well	C-8 hour	T-8 hour
A1	1.0	0.79	C9	1.0	0.88	F5	1.0	1.61
A2	1.0	3.01	C10	1.0	6.97	F6	1.0	1.48
A3	1.0	9.34	C11	1.0	NA*	F7	1.0	10.30
A4	1.0	1.13	C12	1.0	4.69	F8	1.0	3.79
A5	1.0	2.17	D1	1.0	1.99	F9	1.0	0.75
A6	1.0	8.64	D2	1.0	0.88	F10	1.0	2.38
A7	1.0	2.83	D3	1.0	3.20	F11	1.0	1.56
A8	1.0	19.49	D4	1.0	28.48	F12	1.0	3.02
A9	1.0	1.81	D5	1.0	3.32	G1	1.0	1.10
A10	1.0	7.22	D6	1.0	1.28	G2	1.0	4.83
A11	1.0	1.25	D7	1.0	1.10	G3	1.0	5.10
A12	1.0	1.36	D8	1.0	2.70	G4	1.0	6.63
B1	1.0	13.86	D9	1.0	3.30	G5	1.0	1.53
B2	1.0	2.99	D10	1.0	5.42	G6	1.0	1.08
B3	1.0	69.31	D11	1.0	5.47	G7	1.0	1.06
B4	1.0	4.15	D12	1.0	66.12	G8	1.0	0.77
B5	1.0	0.65	E1	1.0	0.53	G9	1.0	3.57
B6	1.0	1.77	E2	1.0	0.74	G10	1.0	2.25
B7	1.0	0.71	E3	1.0	9.39	G11	1.0	2.09
B8	1.0	5.15	E4	1.0	0.59	G12	1.0	0.81
B9	1.0	2.13	E5	1.0	1.72	H1	1.0	4.33
B10	1.0	13.32	E6	1.0	39.23	H2	1.0	2.59
B11	1.0	0.85	E7	1.0	9.92	H3	1.0	2.77
B12	1.0	2.33	E8	1.0	3.64	H4	1.0	0.93
C1	1.0	7.29	E9	1.0	9.08	H5	1.0	51.88
C2	1.0	1.13	E10	1.0	1.46	H6	1.0	6.93
C3	1.0	1.28	E11	1.0	0.64	H7	1.0	1879.33
C4	1.0	3.52	E12	1.0	NA*	H8	1.0	0.61
C5	1.0	2.48	F1	1.0	5.42	H9	1.0	171.14
C6	1.0	1.08	F2	1.0	6.21	H10	1.0	1.0
C7	1.0	2.45	F3	1.0	9.22	H11	1.0	1.0
C8	1.0	2.95	F4	1.0	0.75	H12	1.0	1.0

\*NA-No Amplification

**Table 2. Gene Response to Pathogen Infection.** Data in this table lists fold induction values that were calculated from the threshold cycles ( $C_T$ ) shown in Figure 5. Fold induction was calculated by the equation  $d(F)=2^{-(C_T)}$ , where the  $d(C_T)$  is the  $C_T$  from untreated sample minus  $C_T$  from pathogen-treated sample. The fold induction value for each gene was then normalized against the fold induction of the housekeeping genes.

The cut-off for the regulation of genes was determined by the normalized fold induction values. Up-regulated genes have a fold induction > 2.5, down-regulated genes have a fold induction less than 0.5, and no change was indicated by a fold induction value between 0.5-2.5. Samples labeled NA represent no amplification. C is the control plant relative to T treatment, respectively. The yellow highlights indicate the genes that were up-regulated.

## Conclusions

- The primer library for *Arabidopsis* genes is suitable for expression analysis of defense-related study with high efficiency and reproducibility
- Many genes included in this primer library are expressed in a pathogen-specific manner and have the potential as a disease-related gene signature for mutant characterization or other disease related research
- The walk-away automated protocol for Quantitative RT-PCR using the Primer Library for *Arabidopsis* Pathogen-Inducible genes represents a high-throughput platform for gene expression studies
  - The entire process was automated from primer library plate dilutions through quantitative RT-PCR reaction setup
  - This method was rapid: 96 samples can be processed in 20 minutes from dilution of the primer library to the set up of the PCR reactions.

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