Plant Molecular Biology Reporter **16:** 323–339, 1998.

Protocols

Multiplex Titration RT-PCR: Rapid Determination of Gene Expression Patterns for a Large Number of Genes

ANDREAS NEBENFÜHR[1] and TERRI L. LOMAX*
Department of Botany and Plant Pathology and Center for Gene Research & Biotechnology Oregon State University, Corvallis, OR 97331–2902, USA; [1]*Present address: MCD Biology, University of Colorado, Boulder, CO 80501–0347, USA*

Abstract. We have developed an improved method for determination of gene expression levels with RT-PCR. The procedure is rapid and does not require extensive optimization or densitometric analysis. Since the detection of individual transcripts is PCR-based, small amounts of tissue samples are sufficient for the analysis of expression patterns in large gene families. Using this method, we were able to rapidly screen nine members of the *Aux/IAA* family of auxin-responsive genes and identify those genes which vary in message abundance in a tissue- and light-specific manner. While not offering the accuracy of conventional semi-quantitative or competitive RT-PCR, our method allows quick screening of large numbers of genes in a wide range of RNA samples with just a thermal cycler and standard gel analysis equipment.

Key words: *Aux/IAA* genes, gene expression, gene families, RT-PCR, tomato

Abbreviations: *LeIAA*, *Aux/IAA* gene from *Lycopersicon esculentum* Mill.; MTRP, multiplex titration RT-PCR; RPA, RNase protection assay; *RPL2*, ribosomal protein large subunit 2; RT, reverse transcription.

Introduction

It has become apparent that a high proportion of plant genes belong to families, often with many related members. We are studying a family of auxin-regulated genes, the *Aux/IAA* family, in tomato (*Lycopersicon esculentum* Mill.). We have cloned partial genomic and cDNA sequences representing the tomato homologues of this gene family (*LeIAA1–11*; Nebenführ and Lomax, submitted). The proteins encoded by these genes are localized to the nucleus

*Author for correspondence. e-mail: lomaxt@bcc.orst.edu; fax: 541–737–3573; ph: 541–737–5278.

where they may act as transcription factors (Abel and Theologis, 1995). The large number of *Aux/IAA* genes (at least 11 in tomato, at least 25 in *Arabidopsis*; Kim et al., 1997) raises the question of why higher plants require so many auxin-responsive putative transcription factors. This problem is frequently encountered as modern techniques in molecular biology simplify the isolation of closely-related genes (e.g. McDowell et al., 1996b; Oetiker et al., 1997). While it can be expected that genes within a family have similar biochemical functions, it is of great interest to elucidate the biological role of individual family members.

A first step towards characterization of functional redundancy and/or specialization within a gene family can be the determination of expression patterns for individual genes in different tissues, or in the same tissue under different conditions. However, this analysis can quickly become unwieldy, depending on the size of the gene family and the number of RNA samples to be analyzed. Traditional assays for mRNA abundance, such as RNA gel blots or RNase protection assays, require relatively large amounts of RNA, thus ruling them out as the method of choice for low-abundance transcripts or limited tissue availability. In recent years, a number of methods have been developed that combine reverse transcription (RT) of mRNAs with polymerase chain reaction (PCR) to determine transcript concentrations. These methods avoid the need for large amounts of RNA and offer sensitive non-radioactive detection in small tissue samples (Siebert and Larrick, 1992). Quantitative (or competitive) RT-PCR (Q-RT-PCR), while offering the highest accuracy of these methods, requires the construction of an appropriate competitor template that should be included in the RT-reaction at several concentrations (Riedy et al., 1995). Construction of such a competitor can be time consuming and the need for several RT-reactions makes the procedure expensive.

In contrast, semi-quantitative RT-PCR measures transcript levels based on the band intensity of amplification products relative to an internal control (such as ubiquitin or actin) and does not require the synthesis of a competitor. However, to obtain accurate readings the amplification reaction must be terminated before it reaches a plateau, thus requiring that several control reactions be run before the experiment (Kinoshita et al., 1993). Another semi-quantitative RT-PCR approach is to perform amplification on a dilution series of the cDNA template. The dilution at which the template becomes limiting for PCR can then be used as an indicator of transcript abundance (McDowell et al., 1996a). In the absence of an internal control to measure the efficiency of reverse transcription and amplification, it is necessary to quantify cDNA concentrations of the samples to allow comparative statements to be made.

We have developed a modification of this method which avoids the need for quantification of small amounts of cDNA by including a control primer

pair in every reaction. Our technique does not require the control reactions or densitometric analyses necessary for standard semi-quantitative RT-PCR, thus greatly facilitating the screening of a large number of genes in a large number of small RNA samples. The method described here is also robust enough to allow inclusion of several primer pairs per reaction, thus increasing the efficiency dramatically. We call this method MTRP for Multiplex Titration RT-PCR. While the relative expression levels determined with this method do not offer the reliability of Q-RT-PCR, they can be used to quickly identify family members with interesting expression patterns for subsequent detailed analysis. We have applied this MTRP technique to nine members of the *Aux/IAA* gene family of tomato and have found that it can be used to elucidate tissue- and environment-specific expression patterns for certain family members.

Materials and Methods

Plant material

Tomato seeds (*Lycopersicon esculentum* Mill., cv. VFN8) were surface sterilized for 10 min with 20% household bleach, rinsed extensively, and sown onto moist filter paper (Whatman 3MM) in PhytoTrays (Sigma, St. Louis, MO). Germination and growth occurred in a growth chamber at 28 °C in absolute darkness for 5 d. For light treatments, half of the trays were then transferred to a chamber (28 °C) with continuous fluorescent white light for the final day. Seedlings of similar size (2.5–3 cm tall) were selected, tissues were excised with a razor blade, and then immediately frozen in liquid nitrogen. For auxin-treated tissue, hypocotyls of 5-d-old etiolated seedlings were harvested, cut into sections (5 to 10 mm), and pre-incubated 2 h in the dark in a 1% sucrose, 10 mM MES (pH 6.0) solution to deplete endogenous auxin. The sections were then transferred to fresh buffer either with or without 100 μM indole-3-acetic acid (IAA, Sigma), incubated 2 h in darkness, blotted briefly on filter paper, and frozen in liquid nitrogen.

RNA extraction and cDNA synthesis

RNA extraction from small tissue samples (approx. 100 mg) was performed using the TriZOL reagent (Life Technologies, Gaithersburg, PA) according to the manufacturer's directions. RNA was quantified spectrophotometrically. For complementary DNA synthesis, 3 μg of total RNA was incubated with oligo-(dT) primers and modified MMLV reverse transcriptase (SuperScript II; Life Technologies) at 42 °C for 50 min according to the manufacturer's instructions.

RNase protection assays

RNase protection assays were carried out using the RPAII kit (Ambion, Austin, TX) following the manufacturer's instructions and the following modifications: (a) the ^{35}S-labeled probe was gel-purified; (b) hybridization of the probe (150,000 to 200,000 cpm per assay) and sample followed the 'streamlined' protocol; (c) gels were rinsed in distilled water for 20 min before drying. Films were exposed for 7 to 21 days, depending on the amount of RNA loaded into the assays. Developed films were scanned into a computer with a densitometer (Personal Densitometer, Molecular Dynamics) at 100 μm resolution. Band intensities were determined with the Gel Plotting Macros in NIH Image (http://rsb.info.nih.gov/nih-image/Default.html). *LeIAA* band intensities were normalized to the band intensity of the control gene, *RPL2*, which had been included in all hybridization reactions.

MTRP Protocol

Multiplex PCR[1]

- Assemble amplification reactions (final volume, 25 μl) on ice, each containing:
  - cDNA template dilution
  - 200 μM each dNTP
  - 7 mM $MgCl_2$
  - forward and reverse primer pairs for each gene, including the control gene[2]
  - 1 unit *Taq* polymerase (recombinant; Life Technologies)
  - H_2O to final volume of 25 μl.
- Overlay with 20 μl mineral oil (Sigma), and transfer to a preheated block (94 °C) in a thermal cycler.[3]
- PCR cycles:
  - 1 cycle: 94 °C, 1 min
  - 33 cycles: 94 °C, 10 s; 62 °C, 15 s; 72 °C, 30 s
  - Cool samples to 4 °C.

Electrophoresis

- Load 10 μl of amplification products onto a 2.5% agarose gel (including 0.25 μg/ml ethidium bromide, EtBr), TAE buffer.

- Electrophorese at 5 V/cm for approx. 90 min (lower buffer chamber contains EtBr at the same concentration as the gel).[4]
- Visualize and count bands on a UV transilluminator and note the dilution step of the last visible band.
- Take gel pictures with a gel documentation system.[5]

Notes

1. Experiments were repeated twice on independently isolated RNA preparations, and one representative experiment is shown. PCR analysis was rerun on the same cDNA dilution series for most data points with results within the expected variation of MTRPs (see Results).
2. For the experiments shown here, 150 nM (i.e. 3.75 pmol per reaction) was added of each primer (with the exception of RL/RR and L4L/L4R which were used at 50 nM; see Table 1). Primers should be designed such that each has at least two distinguishing bases at each end. The length should be adjusted such that all calculated annealing temperatures are the same (e.g. 64 °C ±2 °C was used here), based on the '2 + 4' rule (AT-pairs contribute 2°, CG-pairs contribute 4°; Wallace, 1979).
3. A Delta Cycler (Ericomp, San Diego, CA) was used.
4. It is essential to keep gel running and staining conditions identical for comparison between experiments.
5. We used a GelPrint Plus (BioPhotonics, Ann Arbor, MI) with a 'white' reading of 100 to 110 (out of 255) and saved the files to disk in TIFF format. Manipulations of the digitized image involved only general adjustments of brightness and contrast to match the visibility of bands on the printout to that observed under UV illumination.

Results

Primer design and specificity

Primers specific to each of nine individual members of the *LeIAA* gene family as well as an internal control (a housekeeping gene, ribosomal protein large subunit 2, *RPL2*; Fleming et al., 1993) were designed with at least two distinguishing bases at the 3′ end, equivalent annealing temperatures (see Materials and Methods), and spanning an intron so that contamination of the cDNA with genomic DNA could readily be detected (Table 1).

Specificity of the primer pairs was tested in two ways. First, we used individual primer pairs for the amplification of cloned gene fragments. Products were only observed in those reactions that contained the gene and its gene-specific primers. No amplification of sequences by any other primer pair was observed (data not shown). Second, the products of both individual and multiplex PCR of cloned fragments were analyzed by single-strand conformational polymorphism (SSCP, Figure 1), which separates denatured DNA fragments on a non-denaturing polyacrylamide gel (Orita et al., 1989). Every fragment typically results in three bands, representing the double-stranded molecule

Table 1. Primers used for gene-specific amplification. In the primer sequences, 'R' stands for purine (A or G). Expected product lengths for cDNA and genomic templates are given in bp. 'Plasmid' gives the name of the corresponding cDNA clone used in Figure 1.

Gene	GenBank accession number	Primer	Sequence (5′ → 3′)	Product length		Plasmid
				cDNA	genomic	
LeIAA1	AF022012	L1L	CCAGGGAGGACAGATGARGA	375	475	p1c
		L1R	TTCAGACCCTTTATACCCTTCC			
LeIAA2	AF022013	L2L	TATCGAAAGAACATTTTAGAAGCG	167	252	p2c
		L2R	ATCCATCTGTTTCTGAATATACAT			
LeIAA3	AF022015	L3L	GATCATACAGGAAAAATCATGTGT	202	296	p3c
		L3R	TCCTTCTCTTTCTGAATACACTC			
LeIAA4	AF022016	L4L	ACACTCTAGCCTCTGCCTCG	227	344	p4c
		L4R	TTAACATATCCTTCCCAGGAGC			
LeIAA5	AF022017	L5L	ACAGGATGAATAGTTTTAATCAATC	295	710	p5c
		L5R	CTTACATGACTTTGTTACTTGTTC			
LeIAA6	AF022018	L6L	CAGGAAAAATACACTGGCTACT	256	780	p6c
		L6R	CATTGAGAAGATCCATCAAGTGA			
LeIAA8	AF022020	L8L	AAAAAACGTGATAGAAAAGTGCAA	174	290	p8c
		L8R	CATAAGCTTGCTTTCACTTTGAG			
LeIAA10	AF022022	L10L	TTCTCAAAAGCTTGATCGAGAG	175	1100	p10c
		L10R	TGAAATCTTTCATTCCTTGGACAA			
LeIAA11	AF022023	L11L	ACAGTTTTAACGGACGTGAAGC	188	390	p11c
		L11R	ACTTATCTGCATCCTCCAATGC			
RPL2	X64562	RL	GGTGACCGTGGTGTCTTTGC	347	1000	pRc
		RR	ACCAACCTTTTGTCCAGGAGGT			

(renatured prior to entry into the gel; filled arrows in Figure 1) and the two complementary single strands (open arrows in Figure 1). Figure 1 shows the banding pattern observed for multiplex PCR on individual plasmids, pools of the respective plasmids, and cDNA derived from auxin-treated hypocotyl segments, respectively. One of the *LeIAA1* primers is based on a published primer (Mito et al., 1996) whose annealing site lies outside our (shorter) cDNA clone. The control product for this primer pair was therefore generated by gene-specific PCR using only LeIAA1-primers on a cDNA template (Figure 1C).

The banding patterns resulting from amplification of cDNA in the presence of four different primer pairs closely matched those from the corresponding cloned fragments. We have observed a few minor amplification products (arrowheads in Figure 1) which have not been characterized further. None of the minor bands could be detected on an ethidium bromide-stained

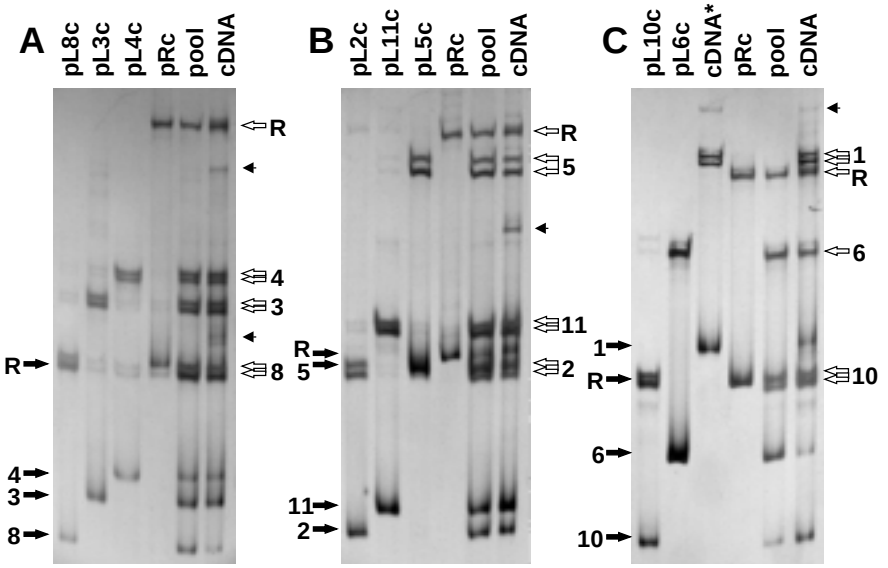

Figure 1. Specificity of primers assessed on a SSCP gel. Multiplex PCR was run with three different pools of four primers each using both cDNA and cloned gene fragments as templates. Primer pools were (A) RL, RR, L4L, L4R, L3L, L3R, L8L, L8R; (B) RL, RR, L5L, L5R, L2L, L2R, L11L, L11R; (C) RL, RR, L1L, L1R, L6L, L6R, L10L, L10R. Lane designations indicate the template: *cDNA* – cDNA derived from RNA of auxin-treated hypocotyls; *pRc* and *p2c -p11c* – individual plasmids containing the respective cDNA fragments; *pool* – plasmid pools (consisting of those plasmids run individually in the same panel); *cDNA** – *LeIAA1*-specific products generated from cDNA in the presence of only L1L and L1R primers (see text). 2 μl of each reaction were denatured in formamide at 94 °C and loaded onto a 5% polyacrylamide/0.25% agarose gel. Electrophoresis was performed at constant 2 W for 4 h. Bands were visualized by silver staining. Open arrows point at single-stranded products, filled arrows denote double-stranded products. Arrowheads identify minor PCR products not predicted by gene-specific PCR.

agarose gel (see Figure 2 and data not shown) with the exception of the unidentified product in pool B (arrowhead in Figure 1B). Even this product could not be visualized at template dilutions greater than 3-fold (data not shown), suggesting that amplification of this fragment did not interfere with amplification and quantification of the major products.

Quantification of template concentrations by titration PCR

The ability of MTRP to determine relative concentrations of transcript for several genes was assessed using the pooled set of primers analyzed in Figure 1A. RNA was isolated from auxin-treated segments of etiolated tomato hypocotyls and cDNA was synthesized from 3 μg of total RNA. Each PCR reaction contained primer pairs specific for *IAA3*, *IAA4*, *IAA8*, and *RPL2*.

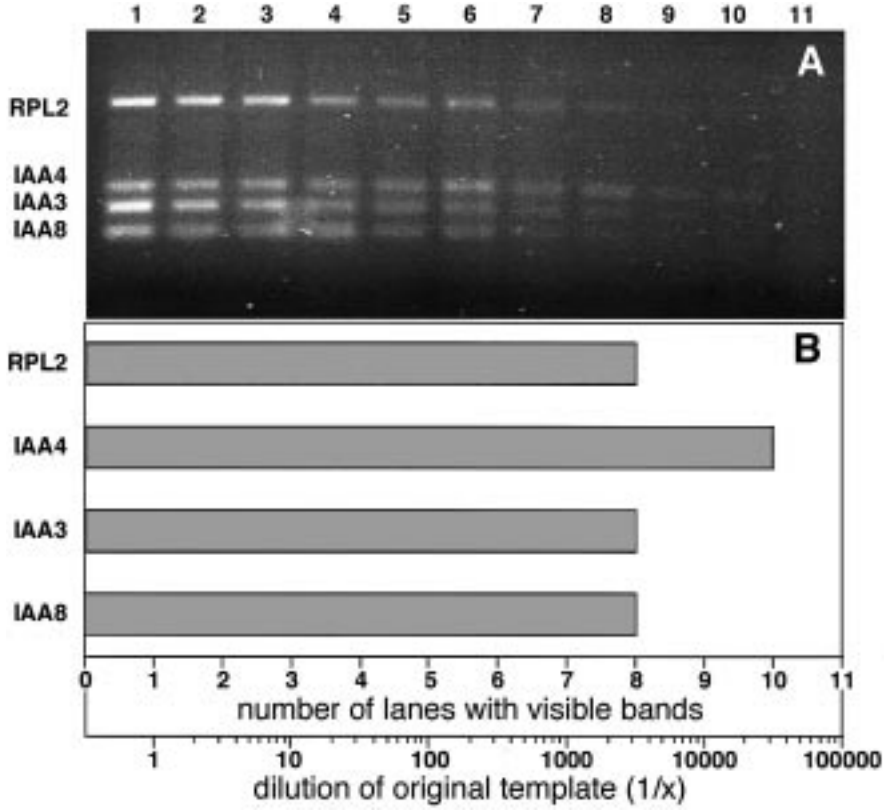

Figure 2. Quantification of gene expression levels by MTRP. (A) Agarose gel electrophoresis of MTRP amplification products. cDNA derived from auxin-treated hypocotyl segments was used as template for the PCR reaction with primer pool A. Lanes correspond to the dilution steps taken from the original template (see panel B). (B) Analysis of panel A. The number of lanes with detectable bands was counted and plotted for all four amplification products. The second x-axis gives the reciprocal (1/x) of the dilution of the original template.

To obtain comparable results, it was necessary to keep the running, staining, and detection conditions consistent between different gels. For estimation of transcript abundance, PCR was run on a dilution series of the original cDNA template and the number of lanes with bands that could be detected on an EtBr-stained agarose gel were counted for all four products (Figure 2A). This number of lanes represents the dilution step at which the template became limiting and therefore provides an indication of the abundance of this particular template species in the original mix (Figure 2B). A 1:3.16-fold dilution series (so that every other step represents ten-fold less template) provided a good compromise between accuracy and economy.

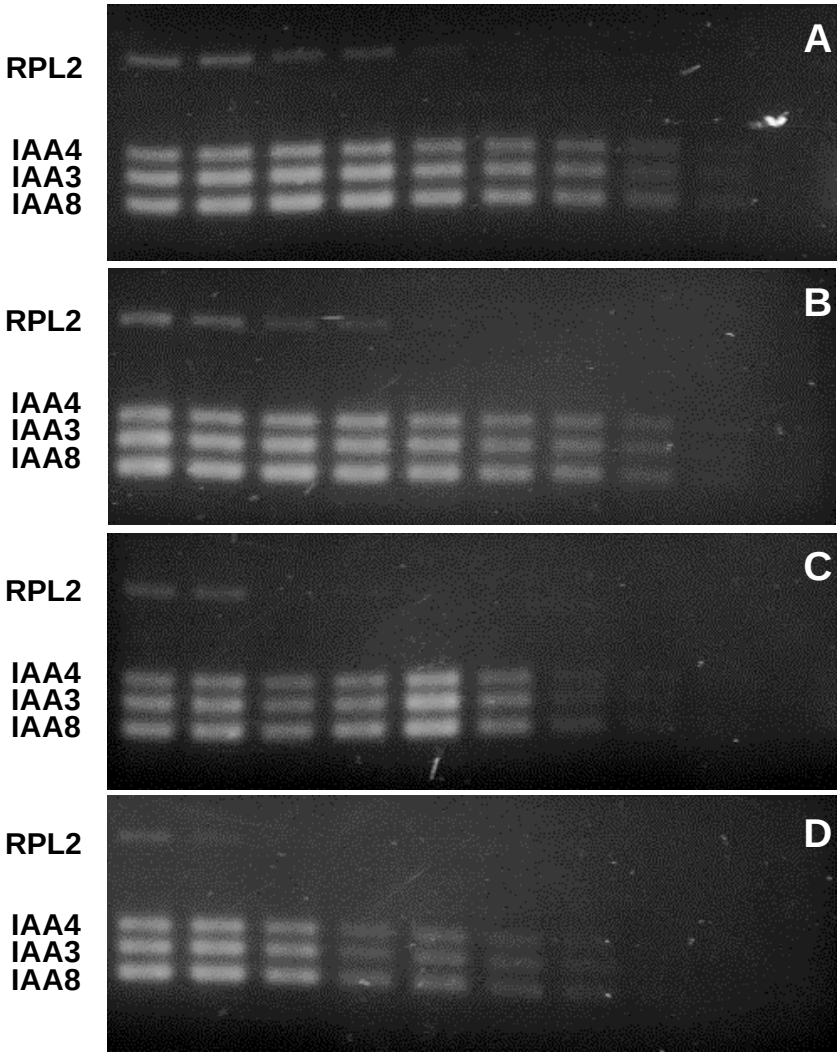

Figure 3. Accuracy of template quantification by multiplex titration PCR. Agarose gel electrophoresis of multiplex amplification products from independent dilution series with different starting concentrations of plasmid templates. Starting concentration of templates were 30 pg (A), 10 pg (B), 3 pg (C), and 1 pg (D) each of plasmids pRc, p4c, p3c, and p8c (see Table 1). Dilution steps of original template are as is Figure 2.

Optimization of the PCR conditions was essential for achieving reliable amplification. In our system, inclusion of several primer pairs in one reaction vessel required higher than usual magnesium concentrations (7 mM) to yield sufficient product for all templates. Also, primer pairs were combined which yielded approximately the same levels of amplification. For example,

Table 2. Effect of varying a single template concentration. Multiplex PCR analysis was run on plasmid mixtures with three plasmid concentrations held constant (pRc, p4c, p3c) while the fourth was varied (p8c). Individual template concentrations are given as pg. The limiting dilution step at which the last visible band was expected (exp) and at which it was observed (obs) is specified as its reciprocal (1/x). Expected dilution steps are based on data from Figure 3.

pRc			p4c			p3c			p8c		
pg	exp	obs	pg	exp	obs	pg	exp	obs	pg	exp	obs
10	30	30	10	3000	3000	10	3000	3000	30	10000	3000
10	30	10	10	3000	3000	10	3000	3000	10	3000	3000
10	30	30	10	3000	3000	10	3000	1000	3	1000	1000
10	30	10	10	3000	1000	10	3000	300	1	300	300

more reproducible data were obtained when the concentrations of the control (*RPL2*) primers (RL/RR) were lowered to 50 nM (one third of the other primers). This resulted in a slight reduction in amplification efficiency for this abundant template, yielding amplification levels that more closely matched those of the *IAA* genes (Figure 2 and data not shown).

Sensitivity, accuracy, and reproducibility

To assess the reliability of this titration multiplex RT-PCR technique for the survey of gene expression levels, tests were performed with known concentrations of plasmids containing cloned gene fragments. First, the sensitivity and accuracy of the assay was addressed by running PCR on a series of template concentrations (30 pg, 10 pg, 3 pg, 1 pg). The number of bands visible on an agarose gel was essentially proportional to the concentration of template (Figure 3). Within the concentration range tested there was a linear relationship between the concentration of a template and the dilution step at which amplification product could no longer be detected, indicating that determination of template abundance is possible. It should be noted that absolute measurements are not possible with this method, as demonstrated by the fact that the L4L/L4R primer pair appeared to have higher amplification efficiency under the conditions used, i.e., this primer pair could be used at lower concentration and still yield the same amount of product (Figure 3). Although a direct comparison of expression levels of different genes therefore is not possible, relative abundance of one transcript can readily be compared between different cDNA samples.

To investigate the possible effects of competition between different templates, the concentration of one plasmid in the mix (p8c) was varied while

Table 3. Reproducibility of MTRP quantification. Multiplex PCR was run on a (1:3.16)-dilution series of a plasmid mix providing 10 pg of template for each primer pair. Data from five independent PCR runs are shown (1–5). In each case, quantification was performed on two independent gels (a, b). Data are presented as the reciprocal of the limiting dilution at which the last visible band could be detected on an EtBr-stained agarose gel.

Experiment	Limiting dilution			
	pRc	p4c	p3c	p8c
1a	30	3000	3000	3000
1b	30	3000	3000	3000
2a	10	1000	1000	1000
2b	10	1000	1000	3000
3a	10	10000	10000	10000
3b	10	10000	3000	10000
4a	30	10000	10000	10000
4b	30	10000	10000	10000
5a	10	3000	1000	3000
5b	3	1000	1000	1000

keeping the others constant (pRc, p4c, p3c). The number of bands observed for the variable template corresponded closely to the amount of template added (Table 2). At the same time, differences in amplification levels for the other amplification products which were in the range of accuracy for this method were observed (see below). We conclude from these data that within the concentration range tested (1 pg to 30 pg per 25 μl reaction), quantification of template abundance is reasonably accurate (within an error of 3-fold) and not affected by the initial concentrations of other amplified fragments.

During the course of these experiments, the results obtained from any one template concentration varied to some extent (3- to 10-fold; Table 3). In general, amplification of the last visible band (i.e., at low template concentrations) was variable, thus limiting the accuracy of the measurement (Saric and Shain, 1997). The results obtained for all amplification products in a pool usually deviated in the same direction (e.g., compare lines 1a and 2a in Table 3). The internal control can provide an alert for these systematic errors, provided that its mRNA concentration is truly constant throughout all RNA samples.

Comparison of MTRP and RPA data

While the MTRP technique does not provide an absolute measure for the abundance of a particular mRNA species in a tissue, it allows comparison of relative mRNA levels in several tissues, or in the same tissue under different conditions. To test the accuracy of quantification of relative expression levels with MTRPs, the results of three independent MTRP analyses were compared with those from RNase protection assays (RPAs). All assays measured the expression of the *LeIAA* genes in independent samples from etiolated hypocotyl segments of tomato seedlings in response to treatment with the plant hormone auxin. Table IV gives the expression levels after auxin-treatment relative to basal expression levels after auxin-depletion (i.e., fold induction by auxin).

The detected induction in response to auxin treatment appeared higher for all genes in the PCR-based experiments in comparison with results from RPAs. This was most pronounced for strongly-induced genes (*LeIAA8*, *10*, *11*). In particular, a difference of 5- to 6-fold in the RPA experiment was represented as a 10- to 30-fold in MTRP (*LeIAA3*, *5*), while a 40-fold difference in RPA was inflated to 3000- to 10 000-fold in MTRP (*LeIAA11*). This result is presumably due to the strong non-linearity of the PCR amplification process. The auxin-responses of weakly-induced genes (*LeIAA1-6*) falls into the range of inherent variability of the MTRP technique (Table 3). However, the simplicity of MTRP lends itself to rapid repeat experiments. Comparison of several MTRP runs could thus be used to distinguish small signals (about 5-fold differences, see Table 4) from random noise, and thus alert the investigator to potentially interesting differences.

MTRP analysis of LeIAA expression in tomato seedlings

When the relative transcript levels of the various *LeIAA* genes were compared in cotyledons, the hook, upper and lower halves of the hypocotyl, the root-shoot node, and the root proper of etiolated seedlings (Figure 4, filled bars), differing expression patterns were observed. Some genes were expressed evenly in all seedling segments (*LeIAA1*), while others showed preferential expression in certain organs. For example, *LeIAA10* was more abundant in hypocotyl segments than in cotyledons. We observed small differences in levels for other genes (e.g., *LeIAA2* for hypocotyl over cotyledons). However, these differences were near the detection limit of MTRP (see above) and therefore should be confirmed with a more quantitative method.

A well-studied effect of auxin is the stimulation of cell elongation. This suggests that expression of genes involved in the elongation response might be down-regulated under conditions that limit cell enlargement. It is known that hypocotyl growth is inhibited by light treatment (Koornneef et al., 1980).

Table 4. Comparison of MTRP and RPA data. Induction of expression of members of the *LeIAA* gene family by 100 μM indole-3-acetic acid in etiolated hypocotyl segments is indicated as changes in transcript abundance relative to auxin-depleted levels. 'RPA': average induction (±SE) from several experiments where RNase protection assays have been used for quantification. 'MTRP1-3': data from three independent auxin treatments and RNA preparations as determined by MTRP analyses.

Gene	Fold induction of expression by auxin			
	RPA	MTRP1	MTRP2	MTRP3
LeIAA1	1.8 ± 0.1	3	10	3
LeIAA2	2.4 ± 1.2	1	3	3
LeIAA3	5.2 ± 1.6	10	100	10
LeIAA4	1.6 ± 0.6	3	3	3
LeIAA5	6.0 ± 2.6	10	10	10
LeIAA6	0.4 ± 0.1	3	10	3
LeIAA8	26.5 ± 8.2	100	100	30
LeIAA10	12.3 ± 5.5	100	30	30
LeIAA11	42.8 ± 11.0	3000	3000	10000

Expression levels of *LeIAA* genes from 5-d-old seedlings treated with continuous white light for the final 24 h (Figure 4, open bars) were compared with results obtained for dark-grown seedlings (Figure 4, filled bars). Expression of *RPL2* and most *LeIAA* genes was not affected under these conditions; however, *LeIAA2* and *10* reproducibly displayed marked reductions in transcript abundance in response to light treatment. This reduction occurred in all tissues for *LeIAA2*, but only in the hypocotyl for *LeIAA10*. It is worth noting that expression of *LeIAA2* and LeIAA10 was reduced even in tissues which did not show greening in response to the light treatment (i.e. lower hypocotyl, node and root).

Discussion

We have developed a simple method for obtaining semi-quantitative measurements of mRNA abundance for a large number of genes. The multiplex titration RT-PCR (MTRP) technique does not require extensive calibration and is therefore well suited for screening large numbers of tissue samples. Quantification by MTRP is based on the fact that template concentrations in

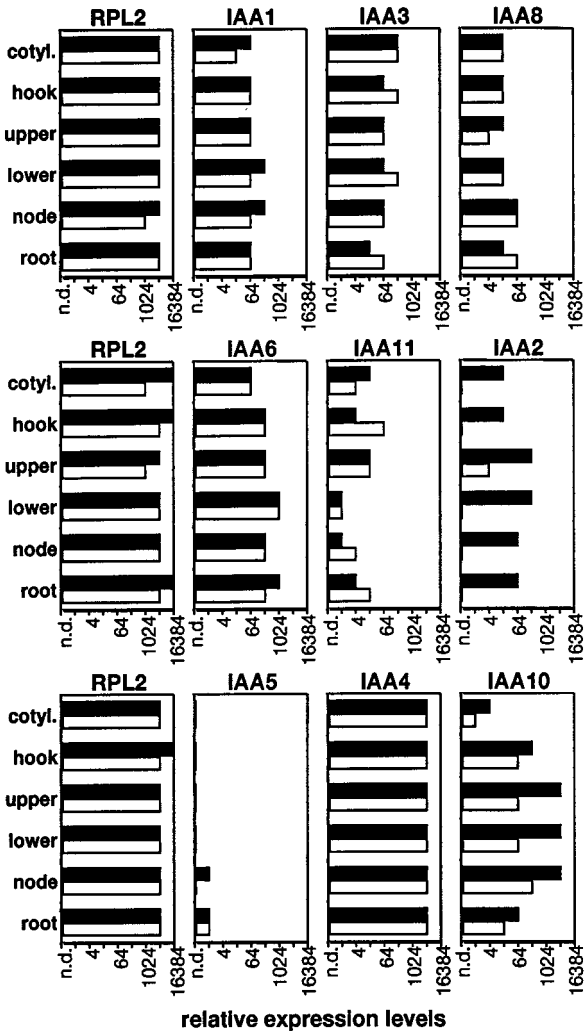

Figure 4. Comparison of *LeIAA* gene expression in dark-grown and light-treated seedlings. Etiolated seedlings were grown in absolute darkness for 5 d (filled bars), light-treated seedlings were transferred after 4 d of growth in darkness to continuous fluorescent white light for 24 h (open bars). Relative expression levels were determined by MTRP and given as the limiting dilution step. Seedling segments are defined in the text; 'upper' and 'lower' refer to the upper and lower half of the hypocotyl, respectively; node is the root-shoot node. Each row of graphs represents one primer pool. Note that in this experiment, different primer pools and dilution conditions (four-fold) were used than those described in Figure 1. Under these conditions, results were comparable to those described earlier, except for *LeIAA5* which was close to the detection limit (data not shown).

a dilution series become limiting for successful amplification. This end-point dilution can be used as an indirect measurement of template abundance, and thus of gene expression levels.

The MTRP method offers four advantages over published approaches to semi-quantitative RT-PCR. First, the amplification reaction does not have to be stopped while both test and control fragments are within the linear phase of amplification, thus eliminating the need for several control reactions for every RNA sample prior to running the actual experiment (e.g., see Fleming et al., 1996). Second, quantification of relative expression levels can be performed quickly under any UV light source and does not require densitometric (or radioactive) analysis of product abundance. Third, it is not necessary to accurately determine the concentration of small amounts of cDNA template since inclusion of an internal standard (such as primers targeting cDNA coding for a housekeeping gene) can be used to detect sample to sample variation. Finally, multiplexing of several primer pairs allows the analysis of three (or more) genes at the same time, thus increasing the efficiency and reducing the cost of the procedure.

Using this technique, we have characterized the expression patterns of members of the auxin-regulated *LeIAA* gene family in a number of tomato tissues at different developmental stages and under different environmental conditions. Five of the genes (*LeIAA1*, *4–6*, *8*) displayed only minor variations in expression levels which could not be resolved unambiguously with the MTRP technique. Some of these differences were reproduced in two RNA samples (e.g., small preferential expression of *LeIAA5* in roots) suggesting that they were not caused by random variation in PCR amplification. However, given the inherent variation of MTRP, it is clear that independent quantification with a different method is warranted to confirm these observations before any conclusions can be drawn.

Two *LeIAA* genes displayed large differences in transcript abundance under different conditions. Both *LeIAA2* and *10* showed marked reduction in expression after seedlings were transferred from constant darkness into light (Figure 4). This response might be involved in inhibition of stem elongation by light. While the physiological relevance of this finding remains to be confirmed, it demonstrates that expression of *LeIAA* genes can be regulated by factors other than auxin. Genes such as *LeIAA2* or *10* may therefore be used to study the interaction of auxin and light in their regulation. Similar comparisons of gene expression with MTRP under a variety of other conditions may be used to rapidly identify the *Aux/IAA* genes which integrate those signals with the auxin response.

The MTRP technique is well suited for determination of expression patterns of larger numbers of genes. In principle, this method can be applied to

any combination of genes, however, the high specificity of the amplification process makes it especially useful for the dissection of closely-related genes within a family, as demonstrated here for members of the *Aux/IAA* family in tomato. Should more genes become available at a later time, it would be easy to assemble another primer pool which could then be run on the already existing dilution series. The high sensitivity of PCR amplification allows the use of small tissue samples (100 mg) which provide ample RNA without getting close to the detection limit for most genes.

Acknowledgements

We want to thank Dr Fred Behringer and TJ White for critical reading of the manuscript. This work was supported by grants from NASA (NAG5 6373) and NSF (IBN-9423651) to TLL.

References

Abel S and Theologis A (1995) A polymorphic bipartite motif signals nuclear targeting of early auxin-inducible proteins related to PS-IAA4 from pea (*Pisum sativum*). Plant J 8: 87–96.

Fleming AJ, Mandel T, Roth I and Kuhlemeier C (1993) The patterns of gene expression in the tomato shoot apical meristem. Plant Cell 5: 297–309.

Fleming AJ, Manzara T, Gruissem W and Kuhlemeier C (1996) Fluorescent imaging of GUS activity and RT-PCR analysis of gene expression in the shoot apical meristem. Plant J 10: 745–754.

Kim J, Harter K and Theologis A (1997) Protein-protein interactions among the Aux/IAA proteins. Proc Natl Acad Sci USA 94: 11786–11791.

Kinoshita T, Imamura J, Nagai H and Shimotohno K (1993) Quantification of gene expression over a wide range by the polymerase chain reaction. Anal Biochem 206: 231–235.

Koornneef M, Rolff E and Spruitt CJP (1980) Genetic control of light-inhibited hypocotyl elongation in *Arabidopsis thaliana* (L.) Heynh. Z Pflanzenphysiol 100: 147–160.

McDowell JM, An Y-Q, Huang S, McKinney EC and Meagher RB (1996a) The Arabidopsis *ACT7* actin gene is expressed in rapidly developing tissues and responds to several external stimuli. Plant Physiol 111: 699–711.

McDowell JM, Huang S, McKinney EC, An Y-Q and Meagher RB (1996b) Structure and evolution of the actin gene family in *Arabidopsis thaliana*. Genetics 142: 587–602.

Mito N, Wimmers LE and Bennett AB (1996) Sugar regulates mRNA abundance of H^+-ATPase gene family members in tomato. Plant Physiol 112: 1229–1236.

Oetiker JH, Olson DC, Shiu OY and Yang SF (1997) Differential induction of seven 1-aminocyclopropane-1-carboxylate synthase genes by elicitor in suspension cultures of tomato (*Lycopersicon esculentum*). Plant Mol Biol 34: 275–286.

Orita M, Iwahana H, Kanazawa H, Hayashi K and Sekiya T (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci USA 86: 1766–1770.

Riedy MC, Timm EA and Stewart CC (1995) Quantitative RT-PCR for measuring gene expression. BioTechniques 18: 70–76.

Saric T and Shain SA (1997) Semi-quantitative RT-PCR: enhancement of assay accuracy and reproducibility. BioTechniques 22: 630–636.
Siebert PD and Larrick JW (1992) Competitive PCR. Nature 359: 557–558.
Wallace RB (1979) Hybridization of synthetic oligonucleotides to phi chi 174 DNA: the effect of single base pair mismatch. Nucleic Acids Res 6: 3543–3557.