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A multiplex reverse transcriptase-polymerase chain reaction method for fluorescence-based semiautomated detection of gene expression in *Arabidopsis thaliana*

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Abstract. A non-radioactive, rapid and sensitive method is presented for the simultaneous detection of several mRNA molecules. The technique is based on conventional first-strand cDNA synthesis by reverse transcriptase, followed by multiplex polymerase chain reaction (PCR) co-amplification of several gene products in a reaction mix containing several primer sets, each including a fluorescently labeled oligonucleotide. The PCR products obtained are finally electrophoresed in a single lane of a polyacrylamide gel, in an automated DNA sequencer controlled by fragment-analysis software. The method has proven useful to efficiently detect nine mRNA transcripts, some of which are low copy number, from small specimens such as single flowers and leaves of *Arabidopsis thaliana* (L.) Heynh. This approach might be easily extended to other biological systems, for developmental and physiological analyses, population studies and diagnosis.

Key words: *Arabidopsis* – Differential gene expression – Multiplex polymerase chain reaction – Transcription detection

The visualization of differential gene expression is a usual part of many contemporary physiological or developmental studies. Although evidence of transcriptional activity has been classically obtained by means of Northern analysis, this technique is becoming progressively substituted by methods based on reverse transcriptase-polymerase chain reaction (RT-PCR; Rappolee et al. 1988). The RT-PCR is more sensitive and less labor and time consuming than a Northern blot, and lacks the problem of low signal-to-noise ratio commonly found when using non-isotopically labeled probes for hybridizing RNA molecules immobilized on a membrane. The co-amplification of several DNA targets in a single reaction mix including several primer pairs is usually termed multiplex PCR (Henegariu et al. 1997). Although it can be used after reverse transcription, in many instances where simultaneous assessment of mRNA presence for several genes is of interest, only a few examples of multiplex RT-PCR have been published, in which the simultaneous amplification of up to four messages has been reported (Chiang 1998; Recchi et al. 1998; Vehaskari et al. 1998).

Arabidopsis thaliana (L.) Heynh. ecotypes used in this work were supplied by the Nottingham *Arabidopsis* Stock Centre (NASC). The *clf-61* mutant was isolated in our lab in a search for ethyl methanesulfonate (EMS)-induced leaf mutants (Berná et al. 1999). Plants were grown as previously described (Ponce et al. 1998). For mRNA isolation, excised rosette leaves or flowers were collected, immediately frozen in liquid nitrogen and stored at −80 °C. Each sample of plant material was weighted prior to being homogenized in 1.5-ml Eppendorf tubes containing 500 µl of Trizol (Gibco/BRL) and incubated for 5 min at room temperature. The RNA was chloroform-extracted, isopropyl alcohol-precipitated and resuspended in H_2O. Genomic DNA was removed by adding 5 units of DNase I (Gibco/BRL) and incubated for 30 min at 37 °C and then for 10 min at 70 °C, to inactivate the enzyme. The RNA was ethanol-precipitated and resuspended in 50 µl of H_2O. First-strand cDNA synthesis was performed with 1 µg of total RNA, which was first incubated for 5 min at 65 °C, immediately put on ice and then incubated for 10 min at 25 °C in the presence of 400 ng of $p(dN)_6$ (Boehringer Mannheim) primers. Reverse transcription was then performed with 200 units of SuperScript II (Gibco/BRL) RT, in a 20-µl reaction mixture containing 0.5 mM of each dNTP, 10 mM DTT, 40 units of RNaseOUT and 4 µl of 5× First Strand buffer (Gibco/BRL), which was incubated for 1 h at 42 °C. The enzyme was inactivated by heating the reaction mix for 15 min at 70 °C.

Synthetic oligonucleotides (Table 1) were purchased from Perkin Elmer-Applied Biosystems UK (Warrington). Each pair of oligonucleotide primers included one labeled with HEX phosphoramidite. Amplifications by PCR were performed in Perkin Elmer 2400 thermocyclers, using 0.2-ml thin-walled tubes, in 5-µl reaction mixes containing 200 µM of each dNTP, 2 mM $MgCl_2$, 0.2 units of Bio*Taq* enzyme (Bioline, London, UK), 0.5 µl of 10× reaction

Abbreviation: PCR = polymerase chain reaction; RT = reverse transcriptase

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Table 1. Primer sets used in this work

Gene	Oligonucleotide sequences (5′ → 3′)		PCR product size (bp)	
	Fluorescently labeled forward primer	Unlabeled reverse primer	cDNA template	Genomic DNA template
AG	ACTCCAACAGGCAATTGATG	TCAAACGGTTGAGATTGCGTTT	299	700
AP1	GGCTTAAGGCTAAGATTGAGC	CTGCTCCTGTTGAGCCCTAA	266	1100
AP2	CGAGTATTTACGATGAGGAACT	GCCGGAAACAGTGAGAATCC	282	630
AP3	CAAGAAACCAAGAGGAAACTGT	AAGAGCGTAAGCACGTGACC	345	1100
APK2a	CATCTGTTACGTTGTTCCACG	AATGGCCCAAAACTACTATCTG	233	≈520
LFY	CCGTGAGTTCCTTCTTCAGG	GTAACAGTGAACGTAGTGTCG	150	1030
CLF	CTTGTCTAGAAGTACTAGTGAA	GGGAAGGTCTTTGGTTTTCTC	420	868
MYB2	TGCTCGTTGGAACCACATCG	GGTGATCATTGACTCCACTTG	381	573
PI	ACATGGCCTCGACAAAGTCC	CACACAGATTGATAAAGACAAAC	323	602

buffer (Bioline) and 0.5 µl of the 20-µl cDNA solution obtained from each sample of plant material. The final concentration of oligonucleotides in the reaction mix was 60 nM, which was achieved by taking 1.2 µl from a master mix containing the 18 oligonucleotides indicated in Table 1, each at a 250 nM concentration. The thermocycling program started with an initial 1.5-min denaturation step at 94 °C, followed by 35 cycles (30 s at 94 °C, 15 s at 55 °C and 1.5 min at 70 °C), and a final 7-min incubation at 72 °C.

Electrophoresis was carried out in a Perkin Elmer ABI PRISM 377 DNA sequencer, using a 4.25% acrylamide-bisacrylamide (29:1, w/w)/6 M urea gel, with 36-cm well-to-read glass plates. The DNA fragment analysis was performed using GENESCAN 2.1 software (Applied Biosystems) as described in the manufacturer's manual. Peak sizes were calculated using the Local Southern Method sizing option. For gel analysis, 2.5 µl of loading buffer, comprising a 5:1:1 (by vol.) mixture of deionized formamide:dye (50 mg/ml blue dextran; 25 mM EDTA pH 8.0):4 nM GeneScan-500 (TAMRA) internal size standard (Perkin Elmer Applied Biosystems), was combined with 1.5 µl of PCR mix. Samples were heated at 94 °C for 3 min immediately prior to gel loading and run for 3 h selecting the GS 36C-2400 module.

We have developed a rapid and reproducible method based on multiplex RT-PCR amplification followed by fluorescence-based semiautomated detection, which allows the simultaneous testing of up to 9 mRNA messages, transcribed from genes of already known sequence. The technique is based on conventional first-strand cDNA synthesis by RT, followed by single-tube multiplex PCR co-amplification of several gene products in a reaction mix containing several primer sets, each including a fluorescently labeled oligonucleotide. In order to differentiate amplifications from either cDNA or contaminating genomic DNA, each primer set was intron spanning (Table 1). The PCR products obtained are finally electrophoresed in a single lane of a polyacrylamide gel, in an automated DNA sequencer controlled by fragment-analysis software. Altogether, oligonucleotide design aimed to allow the production of multiplex PCR-compatible amplification products of non-overlapping sizes, the optimization of PCR conditions, fluorescent labeling of oligonucleotides and the use of fluorescence-based detection in a fragment analyzer make accurate and automated discrimination of minute amounts of amplification products possible without the use of radiolabeling or ethidium bromide staining.

Arabidopsis thaliana flowers and leaves were tested for the presence of transcripts of nine genes (Fig. 1). Assays were performed on a wild-type strain and in a mutant affected in the *CURLY LEAF* (*CLF*) gene (Goodrich et al. 1997). The *clf* mutants display abnormal leaves, which are curled instead of flattened, a phenotype that is known to be associated to the ectopic

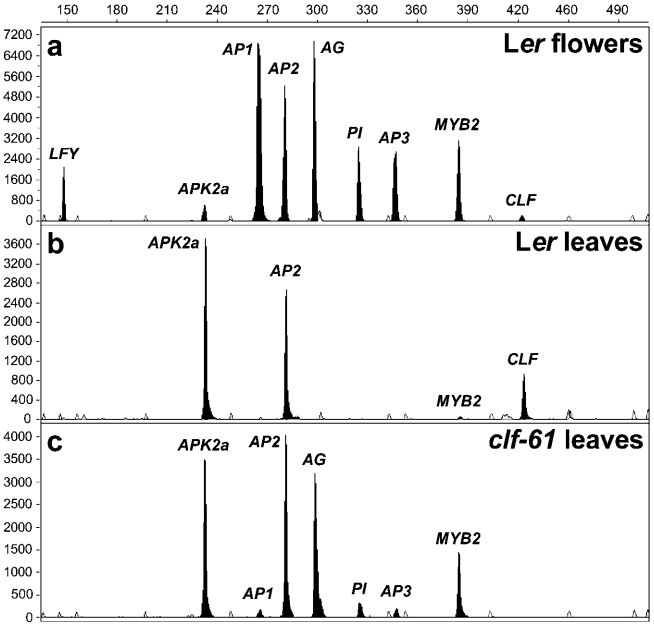

Fig. 1a–c. Electrophoretograms illustrating results of experiments performed following the method presented in this paper on 1 µg of total RNA from the Landsberg *erecta* (*Ler*) wild-type and the *curly leaf-61* (*clf-61*) mutant of *Arabidopsis thaliana*. **a** L*er* flowers (a mixed sample of flower buds and mature flowers). **b** L*er* vegetative leaves. **c** *clf-61* vegetative leaves. The *horizontal* and *vertical* axes indicate, respectively, the size of the electrophoresed molecules (in nucleotides) and the intensity of fluorophore emissions (in arbitrary units of fluorescent signal strength). Each electrophoretogram corresponds to a single gel lane and contains peaks that represent the molecules obtained from the multiplex PCR amplification of cDNA samples obtained from leaves or flowers excised from individual plants 21 d and 5 weeks after sowing, respectively. In the electrophoretogram, produced by the GENESCAN 2.1 software, every peak is denoted with the name of the corresponding gene. *Open peaks* correspond to the internal molecular weight standard, those of cDNAs have been colored in *black*

expression in the leaves of some genes such as *AGAMOUS* (*AG*) and *APETALA3* (*AP3*; Goodrich et al. 1997), whose normal realms of action are the floral organs. The expression of genes at three hierarchical levels of a regulation cascade was assayed: the Polycomb-group gene *CLF* (Goodrich et al. 1997), the MADS-box homeotic gene *AG*, and *APK2a*, which codes for a putative serine/threonine protein kinase (Ito et al. 1997). The *CLF* gene is known to repress *AG* (Goodrich et al. 1997), which in turn is supposed to repress *APK2a* (Ito et al. 1997). Transcription of the floral meristem identity gene *LEAFY* (*LFY*) was also analyzed, together with that of several floral homeotic genes, including members of the MADS-box [*AP3*, *APETALA1* (*AP1*) and *PISTILLATA* (*PI*); Riechmann and Meyerowitz 1997], and AP2/EREBP [*APETALA2* (*AP2*); Riechmann and Meyerowitz 1998] families of transcription factors. Expression of the *MYB2* gene (Urao et al. 1994), which has no known relationship with the specification of floral organ identities, was also studied as an internal control.

Multiplex RT-PCR amplifications were shown to be possible in reaction mixes that included the nine primer pairs required to amplify all the above-mentioned transcripts (Fig. 1), with only minor differences being found in assays performed on total RNA from a given organ from two different wild-type strains, Enkheim-2 (En-2; data not shown) and Landsberg *erecta* (L*er*; Fig. 1a,b). In agreement with previous proposals (Ito et al. 1997), the level of *APK2a* expression in the wild-type leaf (Fig. 1b) was much higher than in the flower (Fig. 1a). As expected, ectopic expression of floral genes was detected in *clf-61* (Fig. 1c) but not in wild-type leaves (Fig. 1b).

Expression patterns revealed by our method are coincidental with those previously obtained by means of classical methods, with two exceptions. First, *AP1* and *PI* gene products were not detected by previous authors in *clf-2* mutant leaves by means of Northern blots (Goodrich et al. 1997), while we clearly detected them in *clf-61* leaf tissues. This may be explained by allele specificity, although it may also be due to a higher sensitivity of our technique. Second, our results, in contrast to those of previous authors (Ito et al. 1997), suggest that the activity of *AG* is not sufficient to repress *APK2a*, which is expressed in the leaves of *clf-61* at similar levels irrespective of the presence of *AG* transcripts.

The method presented here allows one to determine whether or not the transcripts are expressed in a given tissue or organ, even from samples as small as a single *Arabidopsis thaliana* leaf (1–2 mg). Repeated determinations of the same samples and determinations of samples from different individuals of the same genotype and age yielded consistent results, with the only exceptions of *CLF* and *MYB2,* which were somewhat variable. The method is not only reproducible but also sensitive, since several of the messages tested code for transcription factors and are known to be low copy number.

The sensitivity of the procedure presented here makes it easy to predict that further refinements might make it useful even for single cells, as well as quantitative, in order to facilitate studies on variations in the levels of gene expression. It can be easily extended to other biological systems, for developmental and physiological analyses, population studies and diagnosis.

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