High Throughput Cellular Localization of Specific Plant mRNAs by Liquid-Phase in Situ Reverse Transcription-Polymerase Chain Reaction of Tissue Sections

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Advances in high throughput DNA sequencing and bioinformatic gene discovery far outpace our ability to analyze gene function, necessitating development of more efficient means to examine expression at the cellular level. Here we present a polymerase chain reaction-based method to detect mRNA species in situ in which essentially all of the steps are carried out in liquid phase in a 96-well microtiter tray and only the final signal detection is performed on a microscope slide. We demonstrate the sensitivity of the method by the cellular localization of mRNA for the Tkn2 transcription factor in a wide variety of plant tissues, and its selectivity in discriminating a single gene family member by the in situ localization of rbcs3 transcripts. Furthermore, we demonstrate the utility of the in-well in situ method in detecting FDL and IFL1 transcripts in Arabidopsis sections, thus establishing the method as a tool to determine spatial expression pattern of sequences obtained from genomic sequencing projects. Being amenable to robotic processing, in-well reverse transcription-polymerase chain reaction permits a great enhancement in the number of tissue samples that can be processed. Consequently, this method may become a powerful tool for functional genomics studies, permitting the cellular site of transcription of large numbers of sequences obtained from databases to be rapidly established.

With the advent of high throughput DNA sequencing, the rate of gene discovery has begun to far outpace our ability to understand gene function, and new techniques are especially needed to examine expression at the cellular level. Recent advances based on probing microarrays of cloned or PCR-derived sequences (Brown and Botstein, 1999; Ekins and Chu, 1999) or gene chips constructed from oligonucleotides (Lipshutz et al., 1999) permit the temporal expression patterns of hundreds or thousands of genes to be simultaneously examined. In simple tissues such as cultured cell lines or single celled organisms where many or all of the cells might be considered transcriptionally equivalent, DNA array technologies are especially powerful, particularly for revealing changes in mRNA pools following external stimulation, such as serum addition (Iyer et al., 1999), at various cellular or developmental stages (Cho et al., 1998), or in various genetic backgrounds (Hohstege et al., 1998). In multicellular organisms such as Arabidopsis for which abundant genomic and genetic tools have been developed (Somerville and Somerville, 1999; Terryn et al., 1999), it is likely that array approaches will provide detailed information on specific genes and pathways. However, the signals detected in array experiments reflect only the average transcript level in the cells that comprise the target tissue. Furthermore, these experiments give little or no information on the spatial distribution of transcripts within the target tissue. Localization of specific gene expression to particular cell and tissue types is a necessary prerequisite in understanding the function of genes, and will likely become more important as the pace of gene discovery continues to accelerate.

In the study of plants in particular two general approaches have been developed to localize gene expression to the cellular level, and are widely used. One strategy is based on expression of a transgenic reporter such as β-glucuronidase (GUS) or the green fluorescent protein under the control of a cloned version of the presumptive promoter of the gene being examined (Jefferson et al., 1987; Suter-Crazzolara et al., 1995); the spatial and temporal expression of the reporter is assumed to be reflective of the endogenous gene. However because the site of genomic insertion of each transgene is unique, a large number of plants must be examined to eliminate expression variability attributed to sequences (and perhaps chromatin structure) around the insertion point. Furthermore, plant genes have been found in which the promoter extends into the transcribed region (Sieburth and Meyerowitz, 1997), thus being excluded from typical reporter constructs. It also appears that reporter genes themselves are able to influence the transcription patterns of promoters-forward.
(Uknes et al., 1993). Finally, the cellular half-life of reporter gene transcripts and proteins can be expected to differ from those of the gene under examination. The GUS protein is particularly stable (Jefferson et al., 1987) and can persist in cells long after transcription has ceased. For these and other reasons detailed by Taylor (1997), promoter-fusion analysis alone provides insufficient proof of cellular localization of gene expression, and such data alone are unacceptable to certain journals (Taylor, 1997).

The alternative and preferable approach to localize gene expression to the cellular level is by direct detection of transcripts in situ. For plant tissues the standard approach is to challenge tissue sections with a labeled probe (Jackson, 1991; McFadden, 1995). Briefly, tissue is fixed, dehydrated through an ethanol series, embedded in paraffin wax, and sections are cut and mounted on a microscope slide. Samples are cleared (dewaxed), rehydrated through an ethanol series, and subjected to partial proteolysis to make mRNA targets accessible to the probe. Target transcripts are detected by Watson-Crick base pairing to radio- or chemically-labeled probes following standard prehybridization, hybridization, and washing protocols (McFadden, 1995), all performed on the slide. Methods have been developed that incorporate PCR amplification to increase sensitivity and specificity of target detection. Originally developed to reveal DNA targets in situ, such as the presence of lentiviral DNA in infected mammalian cells (Haase et al., 1990), incorporation of a reverse transcription (RT) step prior to PCR has permitted sensitive detection of transcripts in situ in animal (Nuovo, 1992) and plant cells (Johansen, 1997). Various steps in tissue preparation and PCR (including sequential pectinase, proteinase, and DNase digestion) have been optimized for in situ RT-PCR (Nuovo, 1996), and several companies manufacture devices and supplies (such as special microscope slides) that permit the necessary thermal cycling of slide-mounted specimens.

Although current methods for the in situ detection of plant transcripts are able to yield high quality data, these methods are not well suited to high throughput screening, no matter whether hybridization or PCR is used for detection. First, the number of sections that can be mounted on individual slides is limited, especially when slides with special chambers for subsequent PCR are used. Second, although all the specimens on a single slide will be subjected identically to subsequent steps (i.e. as a batch), samples for which different treatments are desired require separate slides. This becomes particularly cumbersome when performing the various controls required for RT-PCR, and prohibits direct side-by-side comparison of adjacent tissue sections probed differently. Finally, the histological constraint of having to cut sections of fixed, dehydrated, and paraffin-embedded tissue, and to perform the subsequent steps that typically take several days, limits the numbers and types of analyses that can be performed. Furthermore, although clearly written and detailed instructions exist (Jackson, 1991; McFadden, 1995), our experience has been that producing high-quality wax sections is far from trivial, and is not a technique that is easily self-taught.

As an alternative to cutting sections, methods to detect mRNAs in whole-mount specimens by in situ hybridization have been developed, and these have proven useful for examining broad spatial patterns of transcripts in whole Arabidopsis seedlings (Ludevid et al., 1992; Rohde et al., 2000). However, the whole mount in situ methods have some clear limitations. Without some independent way to identify particular cells or tissues within the whole mount, it is not possible to unambiguously assign cellular identity to the hybridization signal. Furthermore, these methods require diffusion of large molecules through complex structures, and it is not clear how appropriate controls for tissue or cell-specific variations in diffusion rate can be designed. Nevertheless, for some samples, whole mount methods might represent a powerful prescreening tool to further enhance the throughput of the method presented here.

We have developed a PCR-based method to detect mRNA species in situ in tissue sections in which virtually all of the steps are carried out in liquid phase in a 96-well microtiter tray, and only the final signal detection is performed on a microscope slide. Consequently, it is straightforward to vary the treatments for adjacent specimens simply by varying the composition of each well, or by varying the annealing temperature using a thermal cycler with the capability of creating a thermal gradient. Being liquid based, this procedure is amenable to robotic handling, thus greatly enhancing the number of samples that can be processed. We have found that pretreatment prior to reverse transcription and PCR is unnecessary, thus reducing the overall time of the post-fixation processes to 1 d. Finally, we have found it possible to localize transcripts to discrete cells using sections cut with a microtome (Vibratome series 100 sectioning system, Technical Products International, O’Fallon, MD). We demonstrated the utility of the method in detecting specific transcripts in a variety of plant tissues and in two species (tomato [Lycopersicon esculentum] and Arabidopsis), both of which are widely researched as models. In-well in situ RT-PCR may become a powerful tool for functional genomics studies by providing a high throughput means to establish the cellular site of transcription of genes discovered by bioinformatics.

RESULTS

Tissue Integrity and Sample Uniformity

In traditional methods for in situ detection of mRNAs (hybridization and PCR-based), the fixed tissue sample is securely attached to a solid substrate
(i.e. a microscope slide) during all the post-sectioning manipulations, possibly contributing significantly to maintenance of tissue integrity, especially during thermal cycling. To determine if plant tissues could be processed in liquid phase without significant loss of histological integrity, mock RT-PCR experiments were performed, and the sections were examined in various ways.

Histology of stems from young tomato plants was examined by staining with toluidine blue. At low magnification it was clear that thermal cycling (Fig. 1A) did not appreciably alter the appearance of the various tissues compared with control specimens (Fig. 1B), although treated specimens were more readily stained with toluidine blue. At higher magnification the mock RT-PCR specimen (Fig. 1C) revealed cytology similar to that of the directly stained sections (Fig. 1D). In a second experiment we examined sample-to-sample variability by comparing serial transverse sections of tomato stem under Nomarski optics. Adjacent sections of a series should exhibit predictable changes in the anatomy as one passes through various tissues or organs. In Figure 2, in which sections 1, 3, 5, and 7 of a series progressing in an axillary direction is shown, the emergence of lateral stem outgrowth can be clearly traced, with no apparent artifactual sample-to-sample variability. Together, these results indicate that the rigors of liquid-phase thermal cycling do not appreciably disrupt the anatomy and cytology discernable under the light microscope.

**Detection of Transcripts by in Situ RT-PCR in Microtiter Format**

Because it is present in all transcriptionally-active cells, mapping the presence of ribosomal RNAs provides a convenient tool to assess the ability of the in-well RT-PCR approach to specifically detect transcripts in situ. Using tomato 18S-specific primers, a strong signal was detected in all cells (Fig. 3A). To confirm the specificity of this signal, a reaction was performed in which the amplification primers were omitted, revealing a total absence of cytoplasmic staining (Fig. 3B). Careful examination, however, did reveal weak nuclear staining, possibly reflecting arithmetic amplification from the complex genomic rRNA loci by residual (single) primer used for reverse transcription. Alternatively, incorporation of the digoxigenin-labeled dNTP into nicks introduced during fixation might be sufficient to permit weak staining. Pre-incubation with ribonuclease was sufficient to abolish cytoplasmic staining (Fig. 3C), confirming that the signal truly reflects the presence of transcripts. Again, some weak nuclear staining was apparent. A titration experiment (not shown) revealed that a relatively high concentration (100 μg mL⁻¹) of DNase-free RNase A was required to fully eliminate the signal, presumably reflecting the degree of cross-linking of RNA during fixation. A final control in which Taq DNA polymerase was omitted (Fig. 3D) showed no staining and confirmed that the signal is amplification dependent. Together, these

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**Figure 1.** The effect of thermal cycling on cellular morphology of tissue sections. Longitudinal sections of tomato stem were subject to mock in-well PCR amplification (A and C) and examined following toluidine blue staining. Non-PCR-amplified control (B and D). Scale bars = 100 µm.
results indicate the validity of in situ detection of RNA species in plant tissue sections in a microtiter tray format and confirm the specificity of these reactions.

**Detection of Specific Transcripts**

Having demonstrated the utility of the in-well RT-PCR to detect RNA in situ, we wished to examine the distribution of transcripts from genes expressed in a tissue- and cell-specific manner. Longitudinal sections from tomato stem and floral buds were amplified with primers specific for *Rubisco* (*rbcS3c*) and a class-1 *KNOX* gene (*Tkn2*). Transcripts from the *Rubisco* gene family have collectively been localized by in situ hybridization in floral buds and in shoot apex (Fleming et al., 1993; Fleming and Kuhlemeier, 1994). Furthermore, expression of transgenically-expressed GUS from promoters of certain individual *Rubisco* family members has previously been exam-
rbcS3c. In some instances it was possible to discern altered morphology of these regions, not inconsistent with formation of adventitious root initials (Fig. 4B).

Amplification of longitudinal sections of floral buds with Tkn2 primers gave a strong signal in cells of the meristematic region of the three inner whorls of the floral bud and also in the vascular bundles (Fig. 5A), in accord with the published pattern (Parnis et al., 1997). Also apparent is strong staining of the stamen initials. Significantly, certain cells such as in the emerging sepals were specifically unstained, although a characteristic staining pattern is apparent at the base of, and extending part of the way, into the sepal (Fig. 5A; Parnis et al., 1997); a similar pattern has also been observed for Tkn1 mRNAs (Hareven et al., 1996). Amplification with 18S-specific primers, with which all cells of the bud were strongly stained (Fig. 5B), emphasize this restricted pattern of Tkn2 transcript localization.

To eliminate the possibility that the strong staining observed using Tkn2 primers was reflective of some general feature of the meristem (e.g. being composed of small, presumably transcriptionally-active cells), rather than reflecting the true pattern for Tkn2 transcripts, we used in-well in situ RT-PCR to establish the pattern for rbcS3 in floral buds; it had been demonstrated previously by in situ hybridization and GUS expression that Rubisco transcripts are absent from the meristem dome (Fleming et al., 1993, 1996; Fleming and Kuhlemeier, 1994). Although a small amount of patchy staining was observed in the meristem dome of floral bud with the
rbcS3c primers (Fig. 5C), this is unequivocally distinct from the true meristem pattern obtained with Tkn2 primers, and further demonstrates the power of the in-well method. It is interesting that our detection of rbcS3 transcripts in a restricted set of cells in the meristem differs from previous, hybridization-based in situ localization of Rubisco transcripts, where a strong signal was seen in the sepal primordia and the tissue substanding the meristem (Fleming et al., 1994). This discrepancy likely results from the ability of the in-well in situ RT-PCR to selectively discriminate a single member of a gene family.

Figure 6. In-well in situ RT-PCR detection of Tkn2 transcripts in various tomato tissues (A–F) and detection of FDH (G) and ILF1 (H) transcripts in Arabidopsis tissues. Transcripts were revealed by dark staining in the following tissues: adaxial epidermis (ae); developing carpel, abaxial side (cb); developing carpel, adaxial side (cd); columella cells (cl); collenchyma cells (co); placenta, distal layer (dl); lateral root primordium (lrp); leaflet primordium (lp); root meristem (m); ovule (o); phloem (p); developing petal, abaxial side (pb); developing petal, adaxial side (pd); placenta, proximal layer (pl); placental region (pr); receptacle core (r); root cap cells (rc); vascular system (v); and protoxylem (x). A, D, F, and H scale bars = 100 μm; B, C, and E scale bars = 40 μm; G scale bar = 20 μm.
Detection of Specific Transcripts in Various Tissues and Species

For the in-well in situ method to be a useful and general high throughput tool, it is essential that it can be applied without extensive optimization to a wide range of plant tissues. Furthermore, it should be able to be employed to determine transcript distribution in various plant species, particularly those for which large sequencing projects have been initiated. To confirm these points, we examined Tkn2 transcript distribution in sections from a range of tomato tissues, and also localized transcripts from two Arabidopsis loci.

The spatial expression pattern of Tkn2 is well-established in tomato shoot meristem, young tomato leaves, and floral buds (Chen et al., 1997; Parnis et al., 1997; Janssen et al., 1998a, 1998b). Using in-well in situ RT-PCR (Koltai and Bird, 2000) we confirmed expression of Tkn2 in tomato shoot meristem and found that as previously shown, expression is specifically absent from two of the tunica layers. Amplification of longitudinal sections of gynoecia in tomato floral buds revealed Tkn2 transcripts in the placental region and in the ovules (Fig. 6A), also in strict accord with the published pattern (Janssen et al., 1998b). A signal also was observed in the receptacle core (a tissue not previously examined), and Tkn2 expression was, as previously reported, detected in older floral buds. At higher magnification (Fig. 6B) it is very clear that in the placenta Tkn2 transcripts are restricted to the distal layer, leaving an unstained proximal layer surrounding the ovules; this pattern also is apparent in published, lower magnification images (Janssen et al., 1998b).

In longitudinal sections of young leaf we detected Tkn2 transcripts in leaflet primordia (Fig. 6C) and in a relatively broad band around the vascular system (not shown), also in agreement with the published pattern (Parnis et al., 1997, Janssen et al., 1998a). In longitudinal sections of older, but not fully mature leaf, we also observed very faint Tkn2-specific amplification, both around the vascular system and in the adaxial epidermis (Fig. 6D). Previous northern-blot analyses failed to detect Tkn2 transcripts in mature leaf (Chen et al., 1997; Janssen et al., 1998b), presumably reflecting either the lower sensitivity of this technique compared our PCR-based method, or alternatively, further reduction of Tkn2 message abundance during final leaf maturation. We have previously observed Tkn2 expression in lateral root primordia (Koltai and Bird, 2000) and we show here that individual cells of the developing primordium are readily discernable (Fig. 6E), most apparently at the edge of the primordium. We also found Tkn2 transcripts to be present in the primary root meristem and columella cells (Fig. 6F), but not in other dividing cells in the root such as root cap cells. Together, the spatial patterns we observed for Tkn2 transcripts results confirm that the in-well method is applicable to a wide range of tomato tissues and provides resolution to the level of individual cells.

Because large genomic efforts are currently concentrated on model species other than tomato, we wished to confirm that the in-well in situ method was not specific to tomato, and in particular, that it was suitable for localization of Arabidopsis transcripts. We selected two Arabidopsis genes to test, viz, FIDDELHEAD (FDH) and INTERFASCULAR FIBERLESS 1 (IFL1). FDH, which encodes an α-ketoacyl-coenzyme A synthase was shown previously by in situ hybridization to be expressed in the epidermis of floral organs (Yephremov et al., 1999), and IFL1 transcripts, which encode a member of the homeobox domain ZIP protein family, were localized to sites of interfascicular and vascular differentiation (Zhong and Ye, 1999). By performing in-well in situ RT-PCR on longitudinal sections of young Arabidopsis flowers, we observed FDH transcripts specifically in the outer layer of both adaxial and abaxial sides of developing petal and carpel (Fig. 6G), precisely in accord with the published pattern (Yephremov et al., 1999). Because the spatial distribution of IFL1 transcripts varies in a developmentally, stage-specific manner, we conducted in-well in situ RT-PCR on transverse sections of stem from Arabidopsis plants of various ages. We found that 1- to 2-week-old stems expressed IFL1 in cells adjacent to the developing vascular system (not shown), whereas 12-week-old stems exhibit strong staining in collenchyma cells and in primary phloem cells, and weak, patchy staining in protoxylem (Fig. 6H). Individual collenchyma cells with thickened walls are discernable.

DISCUSSION

In this paper we report a method for in situ localization of mRNAs in sections of plant tissue in which reactions are performed in microtiter trays, permitting the speed and scale of the experiment to be greatly increased compared to current “on slide” methods. Furthermore, except for the initial sectioning and the final colorimetric detection of amplification products, we have reduced the manipulations to a series of liquid handling and thermal cycling steps, which are readily performed by laboratory robots. Even in the absence of automation, performing the reactions in wells permits a single researcher to perform in excess of 96 in situ transcript localizations per day, while varying any or all parameters (such as target gene, tissue type, etc.). We have demonstrated that the technique is applicable to a wide range of organs, tissues, and cell types without specific optimization for each sample, and we have shown that it is an effective method in the two model plants tested (tomato and Arabidopsis). Furthermore, we have demonstrated that the in-well in situ method provides resolution to the single cell level.

The use of PCR to detect transcripts in situ offers several advantages over traditional methods. In par-
ticular PCR affords both increased sensitivity as well as the ability, by the judicious design of primers, to detect transcripts of discrete members of gene families that otherwise might cross-react in a hybridization experiment. We have shown, by detection of \textit{Tkni2} and \textit{rbcs3} transcripts in tomato, and \textit{FDH} and \textit{IFLI} mRNAs in Arabidopsis, that neither of these properties is lost by performing the RT-PCR reactions in liquid phase in microtiter wells, and indeed, that this method is superior to alternative methods. Using a GUS reporter system, Matsuoka and Sanada (1991) demonstrated weak staining around the vascular elements corresponding to \textit{rbcs3} expression, but were unable to confirm this pattern in longitudinal sections, whereas the in-well results we obtained were unequivocal (Fig. 4, A and B). Processing of longitudinal sections by the in-well in situ RT-PCR confirmed the pattern, suggesting that the in-well in situ RT-PCR is more sensitive. We also confirmed that the rigors of liquid-phase thermal cycling do not appreciably disrupt the anatomy or the cytology of the sectioned tissues, or introduce artifactual sample-to-sample variability of sections, as assessed by tolu- idine blue staining, by Nomarski DIC microscopy, and by demonstration of uniform staining with primers for 18S ribosomal transcripts. Thus, the in-well method retains the advantages of in situ RT-PCR while dramatically increasing the number of samples that can be processed.

Previous optimizations of in situ RT-PCR have included an obligate protease and other tissue digestion steps (Nuovo, 1996), presumably to expose nucleic acid within the dense, cross-linked cytoplasm of the fixed cells. We did not find protease digestion to be necessary, and indeed in preliminary experiments that included protease digestion step we observed high, presumably non-specific cytoplasmic staining with the antidigoxigenin antibody (not shown). Nevertheless, we do not rule out the possibility that carefully optimized protease digestion might be beneficial for the detection of very low abundance transcripts. In performing controls to demonstrate the specificity of the RT reaction, we found that a relatively high concentration of RNase A was required to fully digest the fixed RNA, presumably also reflecting the high degree of cross linking of the cellular nucleic acids. Similarly, even overnight incubation with DNase (10 units mL$^{-1}$) did not appreciably reduce staining of the nuclei. Because this staining was \textit{Taq} DNA polymerase-dependant, we interpret this staining to reflect either amplification from the cognate gene, or more likely, non-specific amplification from nicks. Alternatively, nuclear staining may reflect detection of primary transcripts directly at the genomic locus (Vielle-Calzada et al., 1999) or from mRNAs trafficking through the nucleus. However, an absence of cytoplasmic staining in cells exhibiting only nuclear staining implies either transcriptional silence or a specific lack of nuclear export of the specific mRNA in these cells, since we confirmed that cytoplasmic staining truly reflects amplification from RNA. It is interesting that staining of nuclei appeared to be tissue-specific rather than primer pair-specific. Thus, stained nuclei were detected in stems with all primer pairs used, but not in meristems. Perhaps staining of nuclei reflect a cell type-specific accessibility of nDNA or RNA to specific or non-specific amplification.

Whereas nuclear staining of amplified cells may be interpreted as amplification of genomic DNA, control experiments confirmed that cytoplasmic staining reflects amplification only from RNA. Digestion with RNase abolished all cytoplasmic signals, confirming RNA as the sole source of the cytoplasmic staining. Although a weak but detectable cytoplasmic signal was observed when sections were amplified without prior addition of reverse transcriptase (not shown), this presumably results from amplification of transcripts that were reverse-transcribed by the RT activity of \textit{Taq} DNA polymerase (Maudru and Peden, 1997). It is not surprising that the omission of primers from the PCR amplification step abolished cytoplasmic staining, further confirming the specificity of the process.

By permitting the expression profiles of a large number of genes to be rapidly established, the in-well in situ RT-PCR method we have developed will simplify the huge task of assigning function to plant genes by providing a means to couple organ anatomy to transcription patterns. It will enhance better understanding of developmental processes and will permit a breakthrough in the analysis of complex patterns of gene expression.

**MATERIALS AND METHODS**

**Tissue Fixation and Sectioning**

Fresh tomato (\textit{Lycopersicon esculentum} cv Moneymaker) and Arabidopsis (ecotype La-er) organs were cut into small pieces (maximum of 5 \times 2 mm) and immediately fixed at 4°C for 24 h in freshly prepared aqueous FAA (63\% [v/v] ethanol, 5\% [v/v] acetic acid, and 2\% [v/v] formalin). Fixed tissue pieces were washed three times for 10 min each in 63\% (v/v) ethanol and 5\% (v/v) acetic acid and once in phosphate-buffered saline (PBS; 10 mm Na phosphate and 130 mm NaCl, pH 7.5). Samples were embedded by lowering them into molten 5\% (w/v) low-melting point agarose in PBS and cooling them to approximately 45°C in a small Petri plate. Samples were stored for up to 2 weeks at 4°C at this stage.

Small blocks of agarose-containing tissue samples were cut to orient the sample and attached to the specimen block of a Vibratome microtome (Series 1000 Sectioning System, Technical Products International, O’Fallon, MO) using superglue. Sections (40–100 μm) were cut under deionized water and before it sank, each section was either caught with a child’s paint brush or was aspirated into a trimmed Pipettman tip and transferred individually to a well of a
thin-wall polycarbonate 96-well plate (Costar, Cambridge, MA) containing 1,000 units mL⁻¹ RNase inhibitor (Boehringer Mannheim/Hoffmann-La Roche) in 100 μL of sterile water on ice. For very small specimens (up to 1x1 mm), up to 10 sections could be pooled into a single well. As the sections broke through the surface of the water in the Vibrotome sectioning chamber, the agarose typically separated from the tissue. For sections where the agarose did not separate (such as fine, hairy roots), samples were transferred to microtiter wells containing 100 μL of sterile water, and heated to 65°C for 10 min, and washed at 65°C three times with sterile water. Tissue samples sank to the bottom of the wells, permitting convenient removal of solution by aspiration.

In-Well RT and PCR Amplification

The RNase inhibitor was removed from each well by aspiration and replaced with 20 μL of of RT mix (1× RT buffer [Boehringer Mannheim/Hoffmann-La Roche], 1 mm each of dTTP, dCTP, dGTP, and dATP and 0.1 μm gene-specific primer), and the plate was sealed with a sealing mat (thermowell, Costar). The 96-well plate was heated to 65°C for 5 min and then returned to 4°C in a thermocycler with a heated lid (PTC100, MJ Research, Watertown, MA). RNase inhibitor and M-MuLV reverse transcriptase (Boehringer Mannheim/Hoffmann-La Roche) were added to each well to final concentrations of 1,000 units mL⁻¹ and 500 units mL⁻¹, respectively, and incubated at 37°C for 1 h, at 99°C for 5 min, and then returned to 4°C. The wells were washed by aspiration for 5 min in double distilled water. Twenty microliters of PCR master mix (1× PCR buffer [Boehringer Mannheim/Hoffmann-La Roche], 1.5 mm MgCl₂, and 200 μm each dTTP, dCTP, dGTP, and dATP), 10 μm each of the gene-specific primers, and 10 μm digoxigenin-11-dUTP (Boehringer Mannheim/Hoffmann-La Roche) were added to each well. The samples were heated to 70°C for 2 min and 0.2 μL (1 unit) Taq DNA Polymerase (Boehringer Mannheim/Hoffmann-La Roche) was added to each well, followed by 30 PCR cycles (for the primer pairs below: at 92°C for 30 s; at 60°C for 30 s and at 72°C for 1 min).

Primers

Using sequences in GenBank, primer pairs were designed to amplify transcribed portions of the tomato 18S ribosomal RNA gene (accession no. AF179442): 5'−ACCGTGAGAGAAGCAGTGACAGT−3' and 5'−GCCGGGAACATAGTGAAAACCTC−3'. The specificity of each primer pair was established by amplification from tomato and Arabidopsis genomic DNA of a predicted unique fragment whose identity was confirmed by DNA sequencing.

Staining and Detection of PCR Products

Subsequent steps were performed at room temperature. Following PCR the wells were washed twice for 5 min in 1× PBS and blocked for 30 min in 100 μL of freshly prepared blocking buffer (0.1% w/v) acetylated bovine serum albumin [Sigma, St. Louis] in 1× PBS). Alkaline phosphatase conjugated, antidigoxigenin monoclonal antibody (Boehringer Mannheim/Hoffmann-La Roche) was diluted 1:500 in blocking buffer and 50 μL was added to each well and incubated for 1 h. Wells were washed twice for 15 min in 10× washing buffer (0.1 M Tris [Tris(hydroxymethyl)-aminomethane]-Cl and 0.15 M NaCl, pH 9.5).

Sections were gently removed from each well with either a paint brush or by aspiration into a trimmed Pipettman tip, and were stretched on glass superfrost plus, three-chamber microscope slides (MJ Research) in a 50-μL drop of 1× washing buffer containing 150 μg mL⁻¹ 4-Nitro blue tetrazolium chloride and 370 μg mL⁻¹ 5-Bromo-4-chloro-3-indoly-l-phosphate (Boehringer Mannheim/Hoffmann-La Roche). Development of the purple color was monitored by microscopy and stopped by rinsing sections with ultra pure water using a Pasteur pipette. Specimens were covered with a coverslip, sealed with nail polish, and stored at 4°C in a humid box.

To examine tissue integrity, samples were transferred from microtiter wells to a water droplet on a microscope slide, stained with 0.1% (w/v) toluidine blue for 2 min, and washed twice with water.

Sections were observed and photographed on film (Kodachrome 64T, Eastman-Kodak, Rochester, NY) using a microscope (Axiphot, Zeiss, Jena, Germany) equipped with Nomarski Differential Interference Contrast optics.

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