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RESEARCH PAPER

A simple method for obtaining cell-specific cDNA from small numbers of growing root-hair cells in *Arabidopsis thaliana*

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Abstract

A simple and rapid method for cloning specific cDNAs from mRNA populations derived solely from small numbers of root-hair cells is described here. To identify genes expressed during the earliest visible stage of root-hair cell development, cell contents were aspirated from small numbers of *Arabidopsis* root-hair cells at or just before this stage. This material was used to make reusable solid-phase oligo-dT-primed cDNA libraries. To demonstrate that the libraries contained high quality longer cDNAs, a fragment located 2.7 kb from the 3′ end of the cDNA of the single copy root-hair expressed gene *RHD3* was cloned using a nested PCR strategy. This technique was also used to obtain novel gene expression information by cloning the full-length 0.85 kb cDNA of the *Rop2* GTPase from this library. This approach offers a means of cloning larger cDNAs directly from small numbers of growing root-hair cells and, potentially, other epidermal cell types.

Key words: Cell aspiration, complementary DNA, messenger RNA, nested PCR, root hairs.

Introduction

Root-hair cells are a model system for studying the control of cell polarity, and are agriculturally important (Peterson and Farquhar, 1996). Root hairs are highly polarized outgrowths that arise from swellings that form at the apical (root tip) end of root epidermal cells (Dolan *et al*., 1994). Accessing expressed sequence information specifically from root-hair cells would provide a powerful means of understanding which genes are important for root-hair development.

Previously, root-hair enriched cDNA libraries have been made from *Lycopersicon esculentum* (Bucher *et al*., 1997) and *Medicago trunculata* (Covitz *et al*., 1998) by stirring roots in liquid nitrogen to strip extended root hairs. These libraries included large numbers of mature hairs that had stopped growing, and some elongating hairs. However, they did not include material from cells undergoing swelling formation. Thus, any mRNAs present during swelling formation, but not during tip growth, would not have been represented.

More precise information can be obtained by extracting mRNA populations from cell types at specific developmental stages. Although, RT-PCR has been performed successfully using mRNA extracted from single protoplasts (Richert *et al*., 1996) and intact single plant cells (Woo *et al*., 1995; Brandt *et al*., 1999; Laval *et al*., 2002), this technique does not allow repeated use of the same template. An alternative is to construct cell-specific cDNA libraries. This has been done in plants using mRNA purified from around 100 protoplasts derived from micro-dissected maize egg cells (Dresselhaus *et al*., 1994). These libraries were subsequently used to obtain cDNAs of over 1.5 kb (Dresselhaus *et al*., 1996). Cell-specific cDNA has also been prepared from isolated rice sperm cells (Gou *et al*., 2001). Single cell aspiration allows access to the mRNA populations present in specific intact cell types without incurring the inevitable damage associated with micro-dissection. Cell-specific cDNA has been prepared from the aspirated contents of as few as one to ten tomato leaf epidermal cells or guard cells (Karrer *et al*., 1995) and from five barley mesophyll or parenchymatous bundle sheath cells (Gallagher *et al*., 2001). These libraries were

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amplified using PCR and used to detect small cDNA products (under 0.5 kb in length). Previous work has suggested that PCR libraries from tiny numbers of cells are under-represented for larger cDNAs (2–5 kb range; Dresselhaus *et al*., 1994). It is shown here that larger cDNAs can easily be cloned from small numbers of root-hair cells without PCR amplification of the whole cDNA population.

Materials and methods

Plant material and growth conditions

Surface-sterilized *Arabidopsis thaliana* seeds (ecotype Columbia 4) were plated on sterile full-strength Murashige and Skoog agarose medium (Murashige and Skoog 1×, 3% sucrose (w/v), adjusted to pH 5.5–5.7 with KOH, 1% w/v agarose) and chilled for 48 h at 4 °C. Seedlings were germinated and grown in a vertical orientation at 22 °C for 3 d under 24 h light. Prior to cell aspiration, Petri dishes were inverted and lids replaced with laboratory film in order to minimize humidity losses and to prevent hair collapse. Small holes were made in the film for the microscope objective lens and the micropipette.

Micropipette preparation

Borosilicate glass capillaries [O.D. 1.2 mm; I.D. 0.69 mm] (GC120-10, Harvard Apparatus Ltd, Edenbridge, Kent, UK) were used to prepare micropipettes using a PN-3 glass microelectrode puller (Narishige International Ltd, London, UK). To produce apertures of the correct diameter, pulled micropipettes were held at the blunt end and gently dropped onto a sterile Perspex® Petri dish lid to break off the tip. Micropipettes were screened by light microscopy and any with apertures that were too large (inaccurate aspiration) or too small (tip clogs with cell debris) were discarded. Micropipette tips were filled by capillary action from the sharp end with <1 μl mRNA extraction buffer (Dynabeads® mRNA DIRECT™ kit, Dynal AS, Oslo, Norway), with added 0.1% (w/v) Tween-20 (Roche) and 1 U μl^{-1} recombinant RNasin® (Promega UK, Southampton, UK).

Cell aspiration

Tip-filled micropipettes were mounted onto a MO-202 micromanipulator (Narishige). A suitable hair cell was identified and fine control was used to prod the cell to confirm the tip was in the correct position, puncture the cell and aspirate its contents into the tip. Micropipettes were removed from the micromanipulator and attached to a small disposable syringe via a piece of capillary tubing. Positive air pressure from the syringe was used to empty the contents into a 10 μl drop of RNA extraction buffer (also with Tween-20 and RNasin® added) in a sterile 0.5 ml PCR tube (Eppendorf UK Ltd, Cambridge, UK). The empty tip of the micropipette was broken off under the surface of the buffer to ensure that no cell contents were lost. Each PCR tube was then flash-frozen in a dry ice/methanol bath (–70 °C) and transferred to a –70 °C freezer for storage.

mRNA purification

Cell-specific mRNA was purified using the Dynabeads® mRNA DIRECT™ kit (Dynal AS). The manufacturer's instructions were followed throughout. 0.1% (w/v) Tween-20 was added to all buffers to help prevent Dynabeads® sticking to the walls of the PCR tubes (Dynal AS, personal communication). PCR tubes were removed from the –70 °C freezer and kept frozen at –70 °C in a dry ice/methanol bath. As individual tubes were thawed on ice a further 1 U μl^{-1} of recombinant RNasin® was added to each tube.

For whole root mRNA, whole primary roots were frozen at –70 °C on a sterile microscope slide prechilled on a block of solid CO_2, and ground to a fine powder using a prechilled, sterile pestle and mortar. 250 mg powder was gently dispersed and homogenized in 3.44 ml mRNA extraction buffer (Dynal AS), with 0.1% (w/v) Tween-20 added). 300 μl homogenate (approximately 20 mg tissue) was added to 50 μl of Dynabeads® (mini scale preparation) and mRNA was prepared exactly as per the manufacturer's instructions except that 0.1% (w/v) Tween-20 was added to all buffers supplied in the kit.

cDNA synthesis

For cell-specific cDNA libraries, Dynabead®::mRNA complexes were gently resuspended in first strand cDNA synthesis mix (1× first strand buffer, 10 μM DTT, 0.1% Tween-20, 1 U μl^{-1} recombinant RNasin® (Promega UK), 0.5 mM each dNTP, in sterile pure water). After incubation at 42 °C for 2 min, 1 μl SuperScript™ II RNase H^- reverse transcriptase (200 U μl^{-1}) (Invitrogen UK, Paisley, UK) was added. Incubation was for 50 min at 42 °C. The reaction was inactivated by heating at 70 °C for 15 min. The Dynabead®::cDNA complexes were then resuspended in sterile TRIS-EDTA buffer (10 mM TRIS-HCl, 1 mM EDTA, pH 7.6) and stored at 4 °C. For whole root cDNA libraries, Dynabead®::mRNA complexes were used for first strand cDNA synthesis reactions as above, except that incubation was for 60 min at 37 °C, and the reactions were terminated on ice.

Southern analysis

Standard molecular biology techniques were used (Sambrook *et al*., 1989). For Southern analysis, 5 μl of 3′ *RHD3* PCR product was run out on a 1.2% (w/v) agarose gel and blotted onto Hybond N^+ (Amersham Biosciences UK Ltd, Little Chalfont, Bucks, UK). Blots were hybridized to a 3′ *RHD3* genomic DNA PCR product radiolabelled with [α-^{32}-P]dCTP (specific activity $>1\times10^9$ dpm μg^{-1}) using Ready-To-Go DNA labelling beads (Amersham Biosciences UK, Ltd). Blots were exposed to X-OMAT K Film 4528 (Kodak Ltd, Hemel Hempstead, Herts, UK) for 4 h.

Polymerase chain reaction (PCR)

Oligonucleotide primer pairs were designed using Web Primer software (http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer/). All primer pairs were first checked against the *Arabidopsis* DNA database to ensure that there were no non-target sequences similar to each primer. PCR was carried out in a MJ Research PTC-200 Peltier Thermal Cycler (Genetic Research Instrumentation, Braintree, Essex, UK). PCR cycling conditions were: 1 min denaturing at 95 °C, followed by 35 cycles of 30 s denaturing at 95 °C, 30 s annealing at a temperature specific for each primer pair (°C, primary and nested primer pairs, respectively: 3′*RHD3*, 55, 60; 5′*RHD3*, 60, 65; *Rop2*, 55, 65) and 2 min primer extension at 72 °C, followed by 5 min extension at 72 °C. Reaction mixtures contained 1× *Taq* DNA polymerase reaction buffer (Promega) and 0.5 U *Taq* DNA polymerase (Promega), 1.5 mM $MgCl_2$, 0.125 mM each dNTP. 10 μl reaction mixes were made up to volume with pure water and overlaid with a drop of light mineral oil (Sigma–Aldrich Company Ltd, Dorset, UK) sufficient to cover the surface of the mixture. Oligonucleotide primer pairs were as follows (forward and reverse primers, respectively; genomic DNA and cDNA product sizes are shown in bp after each primer pair). Primary: 3′ *RHD3* (5′-GGAGAATTGGCTGTCTATGG; 5′-AACCTCGGATTGAGCTG-TAG, 1475, 855); 5′ *RHD3* (5′-CGCTTGCTCTACCCAGCTTATC; 5′-TTGTGTGCTGGAAGATCCAAG, 1817, 834); *Rop2* (5′-CAA-AGTCATCTATCAACCGC; 5′-CTGTGGACTCGAAAGATTCA, 1738, 1005). Nested: 3′ *RHD3* (5′-GCTCTAGAACGAATGT-GGTCTTTCATACGC; 5′-CGGGATCCAAGCATTGGCAGGGA-CAACTC, 1343, 749); 5′ *RHD3* (5′-CGCGGATCCCGAATT-

TGGGTGATTCTCAG 5′-CCGGAATTCTAGAAATGTCTCCCT-TCACGTC, 1664, 681); *Rop2* (5′-CGCGGATCCCGAATTTGG-GTGATTCTCAG; 5′-CCGGAATTCTAGAAATGTCTCCCTTC-ACGTC, 1587, 853). Nested primers were located approximately 50 bp internal to the first primer. Nested primers also contained unique restriction sites at their 5′ ends to allow directional sub-cloning of cDNAs. Primers (Sigma-Genosys, Pampisford, Cambs, UK) were used at a final concentration of 2.5 ng μl^{-1}. Prior to PCR Dynabeads®::cDNA complexes were washed twice in 1× *Taq* DNA polymerase buffer (Promega). To limit any adverse effect of beads sedimenting under gravity during PCR cycling, beads were resuspended once in each cycle by gently flicking the tube during a brief pause in the denaturation step. Beads were included in the reaction mix for the first ten cycles and then removed by separation with a magnet (Magnetic Particle Separator, Dynal AS) prechilled on ice. 1 µl aliquots of the 10 µl primary reaction were used as template DNA for nested PCRs. Prior to amplification with a new set of PCR primers, DNA on the beads was denatured and washed as described, (Fellman *et al*., 1996).

Cloning of specific cDNA products

PCR products were purified from unwanted secondary products by cutting the bands of interest from agarose gels. PCR products were cloned using the pGEM-T® Vector System I (Promega) following the manufacturer's instructions. 1 µl of each ligation reaction was added to 50 µl sub-cloning efficiency DH5α™ competent cells (Invitrogen UK), following the manufacturer's instructions. Cloned inserts were sequenced using the dideoxy chain termination method: ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, Cheshire, UK) following the manufacturer's instructions. Sequencing reactions were analysed on an ABI 377 DNA sequencer (Applied Biosystems).

Results

Single cell aspiration

To obtain cell contents from growing root-hair cells, seedlings were grown on the surface of sterile medium in a Petri dish. It has previously been shown that even slight damage to a root-hair cell is sufficient to affect the membrane potential in neighbouring cells adversely (Lew, 1991). To avoid sampling wound-induced mRNAs steps were taken to minimize damage to root-hair cells prior to sampling (Fig. 1A, B), and only a single cell was sampled from each dish. Turgor pressure was sufficient to drive cell contents into the micropipette tip (Fig. 1C). As the root epidermis is composed of files of hair cells and non-hair cells it was important to ensure that cell aspiration events were specific to single hair cells. When dye was introduced into an individual target cell, staining was restricted to a single hair-cell ghost (Fig. 1D). This observation is consistent with the symplastic isolation of differentiating root-hair cells reported by Duckett *et al*. (1994). Furthermore, only hair cells that collapsed on aspiration, leaving surrounding cells intact, were used for mRNA purification. The overall success rate of cell aspiration attempts was 64%, (104 successful attempts, 37 unsuccessful attempts). The contents of individual initiating root-hair cells were aspirated into micropipette tips filled with mRNA extraction buffer, and then pipetted into an excess of the same buffer in a 0.5 ml PCR tube (Eppendorf) and frozen at –70 °C until all samples were collected. The time taken from cell aspiration to freezing cell contents at –70 °C was between 20 and 30 s. Root-hair cells were aspirated before swelling formation, and at early and late stages of swelling formation (Fig. 1E), sampling the mRNA population present in hair cells around the time of initiation (Table 1).

mRNA purification, cDNA synthesis and cDNA detection

The term the 'Monte Carlo' effect has been coined to describe aberrant PCR amplification that occurs from very small amounts of complex template DNA, (Karrer *et al*., 1995). To minimize this effect it has been suggested that at least ten plant cells must be pooled to make a single cDNA library. Here the equivalent of 16–24 root-hair cells were pooled prior to mRNA purification and cDNA synthesis (Table 1). To extract poly $(A)^+$ mRNA, pooled cell contents were incubated with oligo dT linked paramagnetic beads (Dynal AS, Oslo, Norway). First strand cDNA synthesis was performed on the beads (Fig. 2). cDNA libraries were also prepared from whole root cDNA (data not shown).

To confirm the presence of cDNA, beads were used as template DNA for PCR. The initial target was a single copy gene that is expressed in root-hair cells, *RHD3* (Wang *et al*., 1997). PCR primers were designed that spanned six introns at the 3′ end of the *RHD3* coding region. The amplification products from genomic DNA (1475 bp) and cDNA (855 bp) were different sizes, and could be used simultaneously to amplify the cDNA product and detect any genomic DNA contamination. Cell-specific PCR resulted in smears (Fig. 3A). To test whether any 3′ *RHD3* PCR products were present in these smears Southern analysis was performed. Autoradiography confirmed that the 3′ *RHD3* cDNA PCR product was present (Fig. 3B).

Cloning of RHD3 *and* Rop2 *sequences from cell-specific cDNA libraries*

cDNA synthesized on paramagnetic beads can be reused as template in different PCRs. It has previously been shown that careful DNA denaturation and washing of these beads avoids cross-contamination during successive amplifications with different sets of primers, (Fellman *et al*., 1996). To show that specific cDNAs could be cloned from these libraries nested PCR primer pairs were designed for two different regions of the *RHD3* cDNA. The *RHD3* transcript is 2.8 kb long (Wang *et al*., 1997). The nested pairs of primers spanned six or nine introns at the 3′ and 5′ ends of the *RHD3* coding region, respectively (Fig. 3C). An important requirement of this technique is first to optimize both primer pairs using readily available cDNA. This step

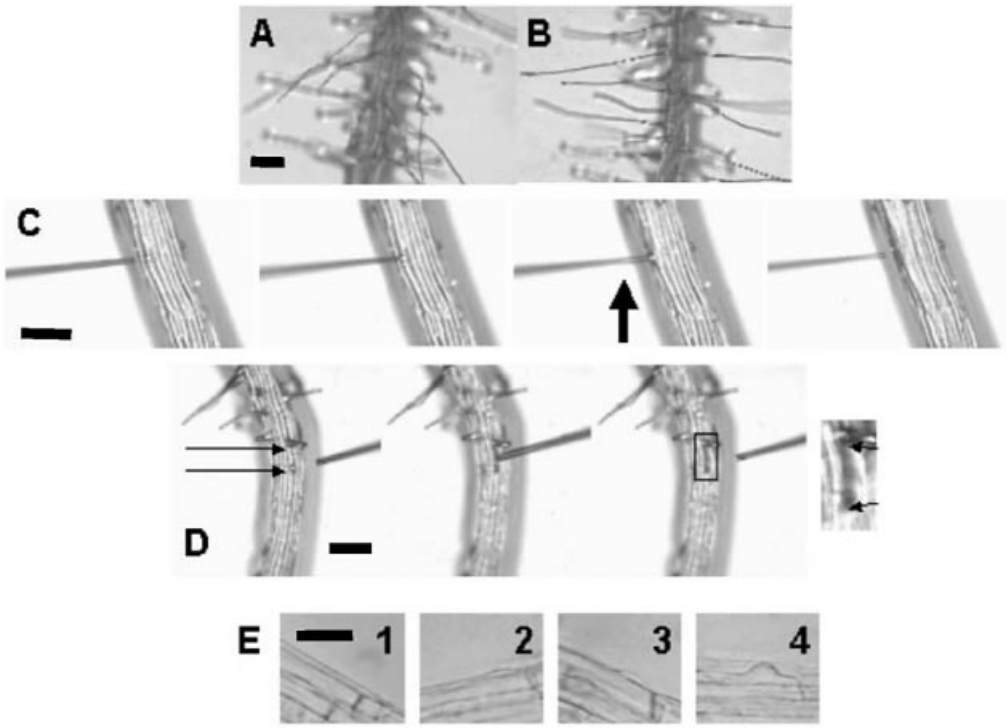


Fig. 1. Cell aspiration of root-hair cells of *Arabidopsis* seedlings growing on solid medium in Petri dishes. (A) Removing the Petri dish lid immediately caused root hairs to collapse. (B) This was prevented by replacing the lid with laboratory film whilst the Petri dish was inverted and cutting holes in the film to allow access for the objective lens and the micropipette. (C) Sequence of images showing the aspiration of a single initiating root-hair cell. Dye (5 mM sulpho rhodamine B) in the micropipette tip is visibly diluted by the aspirated cell contents (arrow). (D) Series of images showing that cell aspiration is probably specific to a single cell. A dye-loaded micropipette was used to puncture and stain one root-hair cell. Arrows mark the end cell walls of the target cell. The area within the white box is shown in close up (right). Staining is limited to the 'ghost' of this single hair cell. Arrows mark the end cell walls of this cell. (E) Cell contents were aspirated from root-hair cells before any visible swelling had formed (1), and during early (2, 3) and late (4) stages of swelling formation. Cells with fully developed swellings were not sampled. Bars in (A–D)=150 μm. Bar in (E)=50 μm.

prevents unnecessary exposure of the cell-specific cDNA libraries to PCR cycling. Here all primer pairs were first optimized using whole root cDNA (data not shown). The 3′ *RHD3* nested PCR product was amplified from cell-specific cDNA libraries 1–4 (Fig. 3D). The 5′ *RHD3* nested PCR product was amplified from library 5, suggesting that this library contained cDNA at least 2.7kb long, (Fig. 3E). Products of expected size were purified from reactions by cutting the bands from agarose gels. These 3′ and 5′ nested PCR products were cloned and sequenced. Using the cloning strategy set out in Fig. 4 both were found to be identical to the appropriate part of the published *RHD3* sequence (data not shown).

To confirm that novel gene expression information could be obtained from these cell-specific cDNA libraries, PCR was performed using nested pairs of primers specific for the *Rop2* GTPase gene (Li *et al*., 2001). Nested primer pairs were designed against the 3′ and 5′ UTRs of the *Rop2* gene. At the time of the experiment it was not known whether *Rop2* was expressed in root-hair cells. It was subsequently shown that *Rop2* is expressed in root-hair cells using *Rop2* promoter::GUS and *in situ* hybridization, (Jones *et al*., 2002). This article describes how the *Rop2* cDNA was cloned from initiating root-hair cells (Fig. 2A in Jones *et al*., 2002) as this method was not described in Jones *et al*. (2002). A single nested PCR product of approximately the expected size for the *Rop2* cDNA was amplified from cell-specific cDNA library 5 (Fig. 3F; Jones *et al*., 2002). This PCR product was cloned and sequenced and found to be identical to the published *Rop2* cDNA sequence (data not shown).

Table 1. *Number of root-hair cells sampled at each stage of development (%)*

Batches 1 and 2 were each divided equally prior to mRNA purification and cDNA synthesis to form libraries 1, 2, and 3, 4, respectively. Batch 3 was used to form library 5. Values in parentheses are the proportions of each cell type in the total (%).

Batch no.	Preinitiation	Early initiation	Late initiation	Total
1	0	1 (3)	32 (97)	33
2	15 (32)	18 (38)	14 (30)	47
3	10 (42)	12 (50)	2 (8)	24

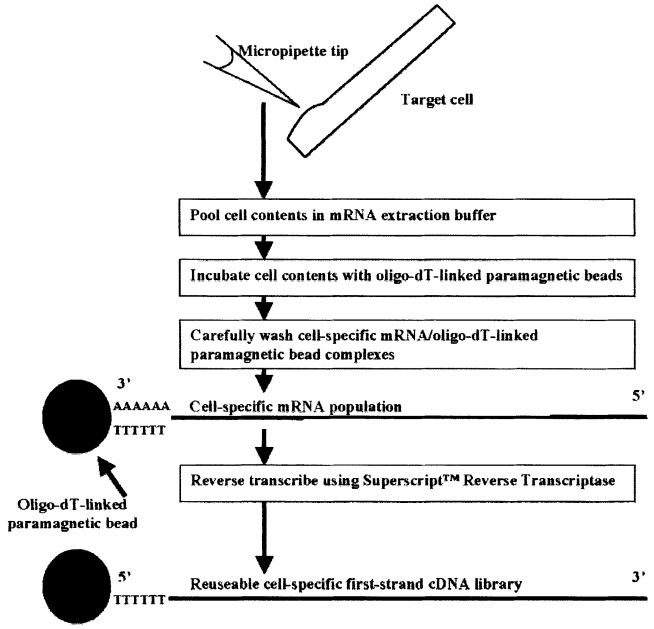


Fig. 2. mRNA purification and cDNA synthesis technique. Cell-specific mRNA was purified directly from aspirated cell contents by incubation with oligo dT linked paramagnetic beads (Dynal AS). Dynabead®:: mRNA complexes were then used directly for first strand cDNA synthesis.

Discussion

A simple, rapid procedure is presented here for constructing cDNA libraries specifically from very small numbers of root-hair cells, and potentially other epidermal cell types, and cloning larger cDNAs directly from these libraries using a gene-specific nested PCR strategy. cDNA fragments were cloned from these libraries, corresponding to the 3′ and 5′ ends of the 2.8 kb *RHD3* cDNA, and the full length *Rop2* cDNA, as previously reported (Jones *et al*., 2002).

The contents of single epidermal cells were removed without prior stress to the plant. This rapid extraction of contents from intact cells suggests that any purified mRNAs should be typical of the population normally present in these cells. These cDNA libraries were not subjected to PCR amplification, which can reduce the representativeness of cDNA libraries derived from small amounts of template cDNA (Karrer *et al*., 1995). Although previous work has suggested that tiny PCR libraries are under-represented for larger cDNAs (Dresselhaus *et al*., 1994), using the technique described here it was easy to obtain cDNA from the extreme 5′-end of the 2.8 kb *RHD3* cDNA. This suggests that there was very little degradation of the *RHD3* mRNA during mRNA purification or cDNA synthesis, and it is likely that other mRNAs represented in these libraries were also not degraded. This technique is both rapid and technically simple. Unlike cell-specific RT PCR, this template cDNA can be reused in multiple PCRs using different primer pairs.

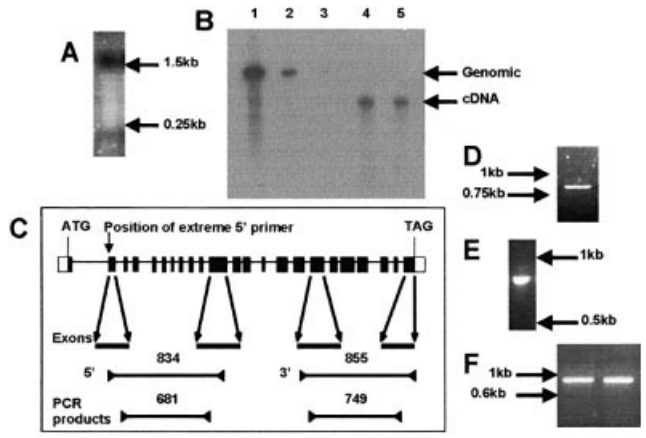
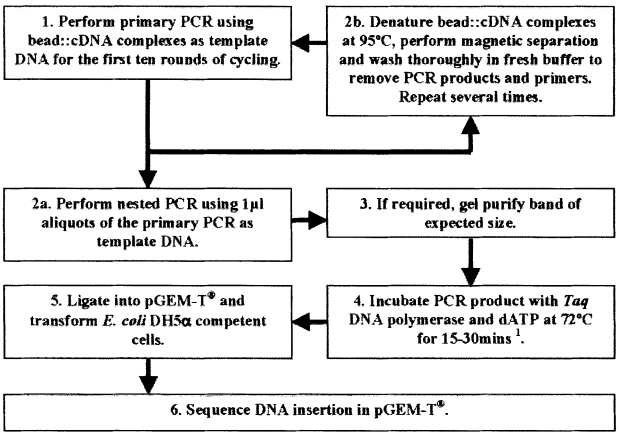


Fig. 3. Detection and cloning of cell-specific cDNAs. (A) PCR on cell-specific cDNA libraries 1–4 using the 3′ *RHD3* primer pair (expected product size 0.855 kb) resulted in smears. The smears shown are from library 4. (B) Autoradiograph of smears probed with a radiolabelled 3′ *RHD3* probe. A specific PCR product of the correct size for the 3′ *RHD3* cDNA is present in lanes 4 and 5. Lanes 1–3 are a dilution series of the 3′ *RHD3* PCR product using genomic DNA as the template. Arrows indicate the expected position of the genomic DNA and cDNA PCR products. (C) Diagram showing the positions of *RHD3* primer sequences (arrowheads) in the *RHD3* gene. Expected PCR product sizes are shown in bp. The 5′ end of the predicted 834 bp 5′ *RHD3* primary PCR product is located 1464 bp upstream from the 5′ end of the predicted 855 bp 3′ *RHD3* primary PCR product. (D) The 3′ *RHD3* nested PCR product (749 bp) amplified from cell-specific cDNA library 4. (E) The 5′ *RHD3* nested PCR product (681 bp) amplified from cell-specific cDNA library 5. (F) The *Rop2* nested PCR product (853 bp) amplified from cell-specific cDNA library 5.



[1] Only required if nested PCR used a DNA polymerase that does not perform template-independent addition of a single A to 3'-ends of PCR products.

Fig. 4. Nested PCR cloning strategy. cDNAs were cloned into the AT vector pGEM-T® (Promega), taking advantage of the template independent addition of a single adenosine to the 3′-end of PCR products by *Taq* DNA polymerase.

Determining whether a candidate mRNA is represented in the library only requires sufficient sequence information to allow the design of a nested pair of PCR primers. This technique could be used to test whether a particular gene is

expressed in a specific cell type before undertaking further molecular work such as promoter::reporter gene fusions or *in situ* hybridization.

Cell-specific cDNA libraries from cells at different stages of root-hair development could provide useful information on changing gene expression patterns during the initiation and polar growth of root hairs. However, plants contain a variety of epidermal cell types. In addition to root-hair cells, guard cells and trichomes are examples of other epidermal cells that could be amenable to study using this approach. In conclusion, it is shown here that accurate expressed sequence information and long cDNA sequences can be obtained from as few as 24 initiating root-hair cells. Cell-specific cDNA could provide a wealth of new expressed sequence information to enhance the molecular understanding of root-hair cells and other epidermal cell types.

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