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Tissue-specific transcriptome profiling of the citrus fruit epidermis and subepidermis using laser capture microdissection

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Abstract

Most studies of the biochemical and regulatory pathways that are associated with, and control, fruit expansion and ripening are based on homogenized bulk tissues, and do not take into consideration the multiplicity of different cell types from which the analytes, be they transcripts, proteins or metabolites, are extracted. Consequently, potentially valuable spatial information is lost and the lower abundance cellular components that are expressed only in certain cell types can be diluted below the level of detection. In this study, laser microdissection (LMD) was used to isolate epidermal and subepidermal cells from green, expanding *Citrus clementina* fruit and their transcriptomes were compared using a 20k citrus cDNA microarray and quantitative real-time PCR. The results show striking differences in gene expression profiles between the two cell types, revealing specific metabolic pathways that can be related to their respective organelle composition and cell wall specialization. Microscopy provided additional evidence of tissue specialization that could be associated with the transcript profiles with distinct differences in organelle and metabolite accumulation. Subepidermis predominant genes are primarily involved in photosynthesis- and energy-related processes, as well as cell wall biosynthesis and restructuring. By contrast, the most epidermis predominant genes are related to the biosynthesis of the cuticle, flavonoids, and defence responses. Furthermore, the epidermis transcript profile showed a high proportion of genes with no known function, supporting the original hypothesis that analysis at the tissue/cell specific levels can promote gene discovery and lead to a better understanding of the specialized contribution of each tissue to fruit physiology.

Key words: Cell wall, citrus, cuticle, epidermis, fruit, laser microdissection, microarray, transcriptome.

Introduction

Fruit expansion and ripening are complex and co-ordinated developmental programmes, involving highly orchestrated biochemical and signalling pathways that mediate cell division, expansion, and differentiation. Many of these have been studied in detail in fleshy fruits, including those underlying changes in size, colour, aroma, texture, disease resistance, and nutritionally important traits (Giovannoni, 2001, 2004; Matas *et al.*, 2009). One feature of all these studies is that any extraction of transcripts, proteins, or

metabolites has been from an homogenized amalgam of the various tissues and cell types that comprise the entire fruit, or parts of the fruit: often the pericarp. Such studies have provided valuable insights into fruit transcriptomes (Alba *et al.*, 2004, 2005; Grimplet *et al.*, 2005; Deluc *et al.*, 2007), proteomes (Sarry *et al.*, 2004; Faurobert *et al.*, 2007; Alós *et al.*, 2008; Muccilli *et al.*, 2009), and metabolomes (Carrari and Fernie, 2006; Carrari *et al.*, 2006; Schauer *et al.*, 2006). However, the mixing of tissues not only prevents resolution

at the cellular level, but also effectively dilutes specific analytes in a broader pool, which might prevent detection when abundance is already fairly low. Some studies have sought to reduce the sample complexity somewhat by targeting specific parts of the fruit, such as analysing peels (Mintz-Oron *et al.*, 2008; Zhang *et al.*, 2008; Ginzberg *et al.*, 2009), which has provided important spatial information, even though the plant material almost invariably still contains varying proportions of multiple cell types.

There is therefore great potential benefit in being able to isolate specific cell types for optimal resolution and a number of approaches have been developed to this end, including cell sorting (Birnbaum *et al.*, 2003, 2005) and micro-mechanical dissection (Brandt *et al.*, 1999, 2003; Thome *et al.*, 2006). However, perhaps the most common is laser microdissection (LMD), where a microscopy assisted laser beam system is used to incise material from tissue sections that are immobilized on a slide (Cornea and Mungenast, 2002). The laser creates an area of isolated cells that can be collected by various methods, such as with an adhesive film (Kerk *et al.*, 2003) in conjunction with a collector tube under the slide (Balestrini *et al.*, 2007), or by catapulting the cells with laser pulses and harvesting them in a collector above the sample (Nakazono *et al.*, 2003). LMD has been applied to profile gene expression in a number of plant vegetative tissues or cell types, including vascular bundles, parenchyma, meristems, incipient leaves, root tissues, and abscission zones (Cai and Lashbrook, 2008; Nelson *et al.*, 2008; Agustí *et al.*, 2009). However, as far as we are aware, it has not yet been used to isolate fruit tissues for subsequent high throughput analyses, but rather to localize a specific transcript type (Raab *et al.*, 2006).

It was investigated whether LMD might provide new insights into fruit development and physiology through the surgical removal and subsequent transcript profiling of specific cell types from the peels of Clemenules mandarin (*Citrus clementina* Hort. Ex Tan.) fruits. Citrus fruits have been used in numerous studies of fruit biochemistry that relate to specific cell or tissue types, such as cuticle composition (Baker and Holloway, 1970; Baker *et al.*, 1975; Nordby and Nagy, 1977) and structure (Garbow and Stark, 1990; Fang *et al.*, 2001; Lai *et al.*, 2003), oil gland formation (Liang *et al.*, 2006) and secondary metabolite accumulation (Sawlha *et al.*, 2009). Moreover, citrus fruits suffer from several commercially important diseases and disorders that are related to the fruit surface (Medeira *et al.*, 1999; Knight *et al.*, 2002). Indeed, isolated citrus fruit peels have been used for anatomical studies of structural irregularities (Safran, 1980), while epicuticular wax morphology and water permeability have been linked to fruit rind physiological disorders known as peel pitting and rind breakdown (Vercher *et al.*, 1994; Agustí *et al.* 2001). A detailed survey of gene expression in specific citrus fruit cell and tissue types therefore not only has great potential importance for a better understanding of the basic aspects of fruit biology, but also has horticultural significance, thereby illustrating the potential value of citrus as a model system in various basic and applied areas of plant research.

In this study, LMD of the epidermal and subepidermal cell layers of Clemenules mandarin fruit, coupled with cDNA microarray analyses, were used to monitor the constituent transcript populations. The results provide insights into cell-type-specific gene expression that can be associated with particular biosynthetic pathways and shed light on differences in core physiological processes between adjacent fruit tissues.

Materials and methods

Plant material

Young, expanding Clemenules mandarin (*Citrus clementina* Hort. Ex Tan.) fruit (approximately 4.7±0.2 cm equatorial diameter) were harvested from adult trees grown in an experimental orchard under normal cultural practices at the Instituto Valenciano de Investigaciones Agrarias Valencia, Spain. Fruits rinds were dissected over a cold surface no more than 30 min after the harvesting and tissues were prepared for sectioning.

Staining and microscopy

To examine the fruit rind morphology, a section of the rind was hand dissected and divided into 5×12 mm pieces. Four pieces from each of four different fruits were pooled for each biological replicate. Four pieces from each biological replicate were immediately snap-frozen in OCT embedding medium (Labonord Cryoblock, France) in Peel-A-Way disposable plastic tissue embedding moulds (Polysciences Inc., Warrington, PA, USA). Cryosections (6, 8, and 12 µm) were cut using a Microm HM550 cryostat (ThermoFisher Scientific, http://www.thermofisher.com) at –26 °C. The sections were transferred to 0.5× adhesive-coated slides using the CryoJane tape-transfer system (Instrumedics, http://www.instrumedics.com) and adhered by UV-crosslinking. Each slide was post-fixed in room-temperature CryoJane aqueous slide fixative [40% glutaraldehyde solution (25% aqueous), 60% CryoJane salt buffer] for 45 s, rinsed gently with distilled water, mounted without staining, or stained with Calcofluor white M2R (Wyeth, http://www.wyeth.com, 0.1% w/v in distilled water). After 1 min of staining the slides were rinsed with water (Gahan, 1984), mounted with a cover slip in DABCO mounting medium and sealed with nail polish.

Bright-field and epifluorescence images were obtained using Zeiss AxioImager A1 microscope (Zeiss, http://www.zeiss.com) equipped with a Zeiss AxioCam MRc colour video camera and ZEISS AXIOVs40 4.6.3.0 software.

Laser microdissection

Cryosections for laser microdissection were prepared as in Agustí *et al.* (2009) with some modifications. From the frozen samples describe above, 10 µm sections were cut with a Leica CM1900 cryostat (Leica Microsystems, Germany) at –20 °C. Cryosections were mounted on PET-membrane-coated stainless steel slides (Leica Microsystems, Germany). Post-fixation included two steps for 15 min in 70% ethanol at –20 °C followed by three xylene steps for 15 min at –20 °C, immediately air-dried, and microdissected. For the isolation and harvesting of the cells from the tissue sections a Leica AS Laser Microdissection system (Leica Microsystems, Inc., Germany) was used to select cells from 16 cryosections for each of the three biological replicates. Cell dissection was performed using the ×40 magnification lens for the epidermal tissue and the ×10 magnification lens for the subepidermal tissue. Microdissected areas were collected in the cap of a 0.5 ml microtube filled with RNA isolation buffer.

RNA isolation, microarray hybridization, and data analysis

Three independent biological replicates were collected for each cell type (epidermis and subepidermis). Approximately 1800 pooled cells were used to obtain total RNA using an RNeasy Micro Kit (Qiagen) following the manufacturer's instructions. Each total RNA sample was used in a two-round RNA amplification procedure performed with the TargetAmp™ 2-Round Aminoallyl-aRNA Amplification Kit (Epicentre Biotechnologies, Madison, WI, USA) following the manufacturer's instructions. The quality of the amplified RNA (aRNA) was evaluated by $OD_{260}:OD_{280}$ measurements.

The microarray hybridization comprised a dye-swap experimental design and the raw microarray data and the protocols used to produce and normalize the data were deposited in the ArrayExpress database (http://www.ebi.ac.uk/microarray-as/ae/) under the accession number E-TABM-954. Each sample was labelled once with Cy3 labelling and once with Cy5, ensuring a dye balance, together with a cDNA citrus microarray (Martinez-Godoy *et al.*, 2008). After hybridization, arrays were scanned with a Scanarray Gx scanner (PerkinElmer Inc. USA) equipped with Scanarray Express software (PerkinElmer Inc. USA) to obtain an appropriate photomultiplier gain ratio for the two channels with a percentage of 1% of saturated spots. GenePix 5 software (Axon Instruments) was used for intensity quantification and data acquisition. Those spots displaying a signal-to-background ratio <2 were discarded together with the flagged spots detected by the software. The quality of the arrays was tested using package arrayQuality (Paquet and Yang, 2008) and the Lowess method was used for normalization. Probes showing significant differential gene expression were identified using the Linear Models in Microarrays (LIMMA) library (Smith, 2004) of the Bioconductor software package (Gentleman *et al.*, 2004). Gene expression differences were only considered significant with *P*-value <0.05 and an *M* contrast cut-off value of ±1, where $M=\log_2(\text{epidermis/subepidermis})$. Positive or negative probe values corresponded to genes preferentially expressed in epidermis or subepidermis samples, respectively. Functional classification of the selected genes was performed using MIPS (Munich Information Center for Protein Sequences, http://mips.helmholtz-muenchen.de/proj/funcatDB/search_main_frame.html) categorization, using the *Arabidopsis* orthologues provided at the Citrus Functional Genomics Project database (http://bioinfo.ibmcp.upv.es/genomics/cfgpDB/getatort.php). InterProScan (Zdobnov and Apweiler, 2001) and SignalP (Bendtsen *et al.*, 2004) algorithms were also used for predicted protein characterization.

Quantitative PCR

Microarray hybridization data for the selected genes were confirmed by real-time RT-PCR (qRT-PCR) analysis with an iQ5 system (Bio-Rad, Hercules, CA). The cDNA samples, as above, were diluted 20-fold with water and 1 μl was used as a template for each 25 μl quantitative PCR reaction, prepared using HotStart-IT SYBR Green qPCR Master Mix (Affymetrix, Santa Clara, CA). For each gene, qPCR reactions were performed in biological triplicates. The sequences of the oligonucleotide primers used are given in Supplementary Table S1 at *JXB* online. The specificity and identity of the products were determined by gel electrophoresis, DNA sequencing, and high resolution melt curve analysis. Data normalization was performed as in Willems *et al.* (2008) with the gene encoding 30S ribosomal protein S9 (array ID: C05141D11SK, contig: aC05141D11SK_c) serving as a constitutive control and assuming PCR efficiency of 1.0 for all genes.

Results

The *C. clementina* fruit is a hesperidium with a rind comprising several distinct tissues (Fig. 1). A prominent structure is the oil gland that appears in tissue sections as
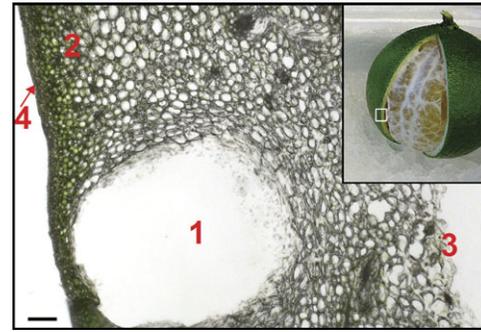

Fig. 1. Light microscopy image showing the anatomy of the *Citrus clementina* fruit rind, highlighting (1) an oil gland, (2) the general area of the flavedo, (3) the albedo area, and (4) epidermis. Insert: one of the fruits used for the sample pooling showing a detached section of the rind and a white rectangle representing the area pictured in the light micrograph. Scale bar=100 μm.

a large hollow space, surrounded by a thin wall. Oil glands span a region from near the surface of the fruit to deep into the albedo, a tissue that is characterized by numerous intercellular airspaces and a characteristic white appearance. By contrast, the flavedo is composed of tightly appressed chromoplast-rich parenchyma cells that contribute the typical green, yellow or orange colour to the citrus fruit. Finally, the epidermis is formed by a single continuous layer of conical cells covered on the outer periclinal wall by a hydrophilic cuticle.

Tissue microdissection and RNA isolation

Importantly, the cryofixation and sectioning procedures used prior to LMD resulted in samples that retained the main anatomical features (Fig. 2A) that are observed in untreated samples (Fig. 1). This indicated that the post-fixation process did not cause major tissue disruption, and the well-preserved differences in cellular morphology between tissue types provided an excellent source of structurally defined material for subsequent LMD. An example is shown in Fig. 2B, where a group of epidermal cells was selected by drawing an encircling line (shown in blue), defining the path that the laser then followed. The dissection resulted in clearly demarked zones of dissected epidermal cells (shown by empty spaces in the post-dissection image; Fig. 2C), while for the capture of the epidermal cells, the adjacent subepidermal cells were dissected by the laser during the capture. Samples of intact subepidermal cells were then selected from the region adjacent to, and one or two cell layers below, the captured epidermal cells (zone 2; Fig. 2C). An example of a single section from which both cell types were harvested is shown in Fig. 2D, highlighting the distinct physical separation between the two tissues. Approximately 1800 cells were captured directly into RNA extraction buffer for each cell type and after two rounds of RNA amplification, totals of approximately 1 μg, 33 μg, and 9 μg of amplified RNA (aRNA) were obtained for the three biological replicates of
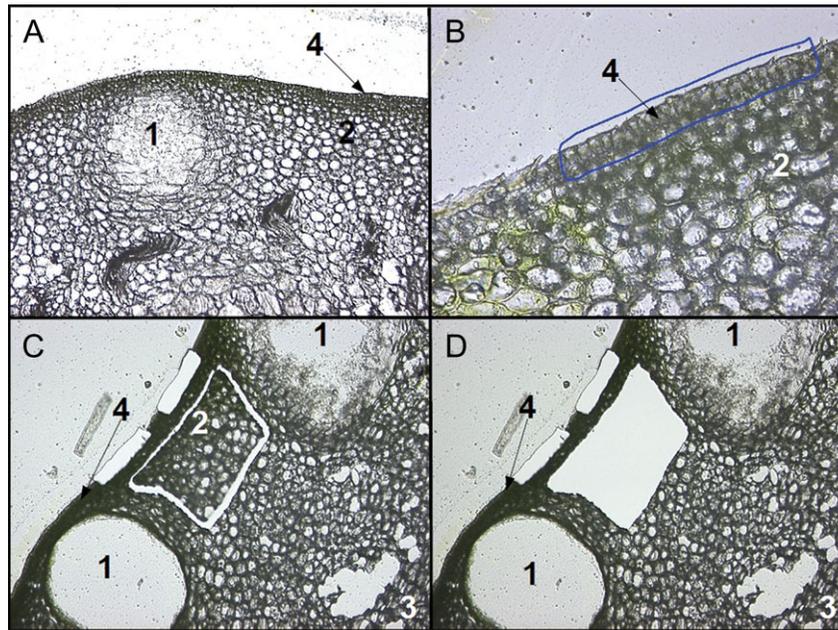
Fig. 2. (A) Light microscopy showing the anatomy of the *Citrus clementina* fruit rind after fixing and sectioning prior to Laser Microdissection (LMD); (B) epidermal cells selected before dissection, (C) fruit section after LMD capture of epidermal cells, also showing laser excised subepidermal cells prior to capture; (D) the same section of subepidermis after the capture. (1) Oil gland, (2) flavedo area, (3) albedo area, and (4) epidermal cells.

the epidermis samples, while the subepidermis sample replicates yielded approximately 1 μg, 86 μg, and 115 μg of aRNA. These amounts of aRNA were sufficient for a two biological replicate/two dye swap design for the microarray hybridization and a three biological replicates design for the quantitative RT-PCR analysis.

Microarray hybridization and gene expression

The aRNA samples were labelled with Cy dyes and used to probe a citrus microarray (Martinez-Godoy *et al.*, 2008) comprising approximately 21 000 putative unigenes from different citrus species and varieties (mainly *C. clementina* cv. Clemenules). The gene annotation and additional information about the libraries used in the array construction and query tools are available at the Citrus Functional Genomics Project database (http://bioinfo.ibmcp.upv.es/genomics/cfgpDB). Based on selection criteria of a 2-fold difference in signal intensity and a *P*-value <0.05, a total of 158 genes were more abundantly expressed in epidermal tissue (see Supplementary Table S2 at *JXB* online), of which 31% currently either have no annotation in the citrus database, or are annotated only as 'expressed protein'. Conversely, 177 genes were predominantly expressed in the subepidermis (see Supplementary Table S3 at *JXB* online), of which 30% had no annotation in the citrus database. From these two datasets, 34% and 29% from epidermis and subepidermis, respectively, had no *Arabidopsis* orthologue. However, it was notable that approximately half of the top 15 most epidermis predominant (EP) genes have no substantial sequence similarity with *Arabidopsis* genes (Table 1), while all but one the subepidermis predominant

(SP) genes have *Arabidopsis* homologues (Table 2). Most of the top 15 annotated EP genes are associated with general lipid metabolism, cutin and wax metabolism, or flavonoid biosynthesis. Furthermore, more than half of the top 15 predicted EP proteins have a putative signal peptide for targeting to the secretory pathway, and/or transmembrane domains. On the other hand, most of the top predicted SP proteins are related to primary cell wall biosynthesis and modification, while other common annotation terms in the larger SP list are associated with energetic processes, photosynthesis and electron transport-chain reactions, transport and sugar or protein biosynthesis.

The expression of a 12 genes (six for each tissue), which collectively showed high, medium, or low (~2.0) fold differences in transcript abundance between the epidermis and subepidermis, was tested by quantitative RT-PCR (qRT-PR) to validate the microarray data (Fig. 3A). In addition, a 30s ribosomal protein gene was used as the control for constitutive expression. Of these genes, only those that resulted in a single amplified PCR product that matched the expected sequence upon resequencing, were used for the comparison. In general, the relative expression level values as determined by PCR agreed with the fold change observed in the microarray (Fig. 3B), with a correlation coefficient of 0.95. All 335 differentially expressed cDNAs were grouped into functional categories according to the Munich Information Center for Protein Sequences (MIPS; Fig. 4). In both sets of differentially expressed genes, the most represented functional class was Binding Proteins, followed by Metabolism. The SP gene set preferentially grouped with the Metabolism, Energy, and Storage Protein categories, while the EP set was highly enriched in

Table 1. List of the top 15 differentially expressed [epidermis (E) versus subepidermis (S)] genes in *C. clementina* fruit epidermal cells

Citrus EST accession number	Fold difference (E/S)	Arabidopsis orthologue	Arabidopsis description	*E*-value	Signal peptide	InlInterProScan domains
C02010G07	1078	AT3G16370	GDSL-motif lipase/hydrolase family protein	1.00E-128	Yes	Lipase GDSL
C06023A05	638	AT3G21090	ABC transporter family protein	2.00E-016	No	Transmembrane regions
C01008D06	477	N/A			No	No hits reported
C20004C04	413	AT2G47240	Similar to long-chain-fatty-acid-CoA ligase,	7.00E-047	No	Acetyl-CoA synthetase-like
C34003G07	380	N/A			Yes	Transmembrane regions
C34007H06	336	N/A			No	No hits reported
C01006A03	285	N/A			No	Transmembrane regions
C04011H06	273	AT4G15093	Catalytic LigB subunit of aromatic ring-opening dioxygenase family	1.00E-082	Yes	LigB aromatic-ring-opening dioxygenase
C16015C11	243	N/A			No	Transmembrane regions
KN0AAP8YE05	174	N/A			Yes	Transmembrane regions
C20005G09	79	AT5G53480	Importin beta-2	1.00E-006	Yes	Transmembrane regions
C06009B08	49	AT5G57800	CER1 protein, putative (WAX2)	1.00E-108	No	G3DSA:3.40.50.720, NAD(P)-binding Rossmann-fold domains
C07008E01	46	N/A			Yes	Transmembrane regions
C31206G08	43	N/A			No	No hits reported
C06023B04	37	AT4G21620	Glycine-rich protein	4.00E-015	Yes	Transmembrane regions

Table 2. List of the top 15 differentially expressed [subepidermis (S) versus epidermis (E)] genes in *C. clementina* fruit subepidermal cells

| Citrus EST accession number	Fold difference (S/E)	Arabidopsis orthologue	Arabidopsis description	*E*-value	Signal peptide	InterProScan domains
C07009G03	892	AT2G32540	Cellulose synthase family protein	1.00E-167	No	Cellulose synthase
C06001A06	477	AT1G69530	Expansin, putative (EXP1)	1.00E-103	Yes	Expansin
C04029A07	254	AT4G19420	Pectinacetylesterase family	7.00E-070	No	Pectinacetylesterase
C08011G02	246	AT5G25610	Dehydration-responsive protein (RD22)	1.00E-008	Yes	BURP, dehydration-responsive protein (RD22)
C01008H04	144	AT3G45040	Phosphatidate cytidylyltransferase protein	2.00E-006	No	
C02013F09	108	AT1G77120	ATADH	1.00E-162	No	Alcohol dehydrogenase GroES-like
C31305G10	88	AT1G17860	Trypsin and protease inhibitor family protein	1.00E-021	Yes	Proteinase inhibitor I3, Kunitz inhibitor ST1-like
C20008D08	76	AT1G48600	Phosphoethanolamine N-methyltransferase 2	3.00E-062	No	*S*-adenosyl-L-methionine-dependent methyltransferase
C20006F05	68	AT3G18000	Phosphoethanolamine N-methyltransferase 1	0	No	*S*-adenosyl-L-methionine-dependent methyltransferase
C06003C09	56	AT3G57520	Alkaline alpha galactosidase	5.00E-061	No	Raffinose synthase
C04011E09	56	AT5G47560	ATSDAT, a tonoplast malate/fumarate transporter.	0	No	Sodium/sulphate symporter, transmembrane regions
C06008E06	51	AT3G21670	Nitrate transporter (NTP3)	4.00E-060	Yes	Proton-dependent oligopeptide transport (POT) family, major facilitator superfamily
KN0AAP5YA02	47	N/A			No	No hits reported
C06019F11	43	AT2G21590	Similar to glucose-1-phosphate adenylyltransferase large subunit 2 (APL2)	2.00E-045	No	glucose-1-phosphate adenylyltransferase, trimeric L pxA-like enzymes, bacterial transferase hexapeptide repeat
C07007D04	41	AT4G15440	Hydroperoxide lyase (HPL1)	4.00E-021	No	No hits reported
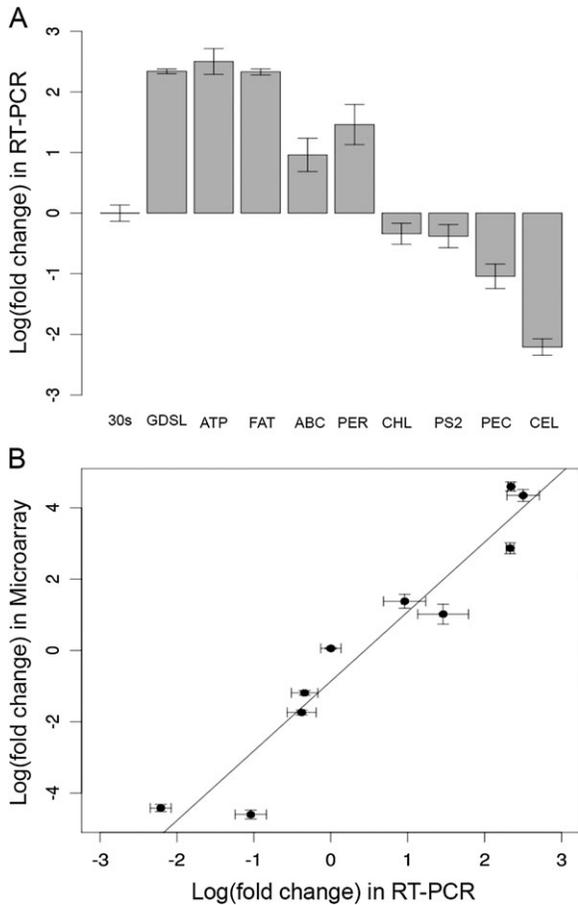
Fig. 3. Microarray gene expression validation by quantitative real-time PCR (qRTPCR). (A) Expression levels of selected genes that are more abundant in the epidermis [GDSL, GDSL-motif lipase/hydrolase (aCL3133Contig1); ATP, ATP-dependent transmembrane transporter, ABC transporter (aC06023A05SK_c); FAT, fatty acid hydroxylase (aCL8782Contig1); ABC, putative ABC transporter (aCL8042Contig1); PER, peroxidase (aCL1534Contig1)], or subepidermis [CHL, chlorophyll a/b binding protein (aCL2421Contig1); PS2, PSI light-harvesting antenna chlorophyll a/b-binding protein (aC05801H09SK_c); PEC, pectinacetylesterase precursor (aCL67Contig6); CEL, putative cellulose synthase (aCL1355Contig1)], as determined by qRTPCR. 30s: Constitutive expression control 30s ribosomal protein (aC05141D11SK_c). (B) Correlation between levels of transcript abundance based on microarray analysis and qRTPCR. The correlation (R2=0.95) was calculated using log-transformed values of the fold change obtained for the nine selected genes.

genes belonging to the Defence, Cellular Transport, and Interaction with the Environment categories.

Microscopic validation of cell-type-specific processes

Among the most notable results of the expression profiling was the prevalence of genes associated with both polysaccharide cell wall metabolism and photosynthesis in the subepidermis, and with cuticle biology in the epidermal cell layer. To investigate this further, Calcofluor staining was used to visualize the cellulosic cell wall, which revealed no

major differences in wall thickness or morphology between the epidermis and subepidermis (Fig. 5A). The differences in the cell layers was, however, marked when the autofluorescence of the sections was examined, since chlorophyll was exclusively seen in the underlying tissues and not the epidermis, while, conversely, the flavonoids gave a distinctive green colour only in the epidermal layer.

Discussion

High throughput gene or protein expression profiling are now commonplace strategies to study complex biological processes and their value is indisputable. However, it can be argued that the accuracy and sensitivity of such approaches are lessened by the fact that typically RNA or protein samples are extracted from bulk organs and tissues, which will frequently result in the mixing of many disparate cell types. An attempt was made to determine whether the isolation of specific cell types by LMD would provide not only a more sensitive means to monitor gene expression, but would also provide new insights into aspects of fruit physiology that are not apparent through a study of homogenized tissues.

It was found that, through the use of careful cryofixation and sectioning, it was possible to obtain tissue sections that were indistinguishable from those that had not been post-fixed and had well-preserved tissue morphology, and so were amenable to LMD. In addition, this procedure yielded sufficient levels of RNA for downstream analysis and although two rounds of RNA amplification were necessary for the array experimental design used here, it has previously been shown that this does not result in a significant relative distortion of the transcript population (Puskas *et al.*, 2002; Nakazono *et al.*, 2003).

Analysis of the microarray data clearly showed that the use of LMD resulted in a substantial increase in resolution and specificity of the gene expression differences over other related approaches that did not generate such pure populations of cell types, such as relying tissue peels, which inevitably contain multiple cell layers (Goudeau *et al.*, 2008; Zhang *et al.*, 2008; Ginzberg *et al.*, 2009). Indeed, differences of up to three orders of magnitude were seen for some genes. This in itself indicates that the harvested cell type pools had a high degree of purity.

As anticipated, the microarray data revealed a high degree of spatial variation in different aspects of fruit physiology and clear cell-type-specific compartmentation of biochemical processes. For example, the single most statistically significant difference between the epidermis and subepidermis is the number of SP genes that are associated with photosynthesis and energy generation. This is in agreement with the presence of chloroplasts in the subepidermal, but not the epidermal cells, as shown in Fig. 5B. This raises an interesting question regarding the provision of energy for the epidermal cells, since they are the site of cuticle biosynthesis, which represents a major carbon sink and considerable energy investment, and yet these cells have
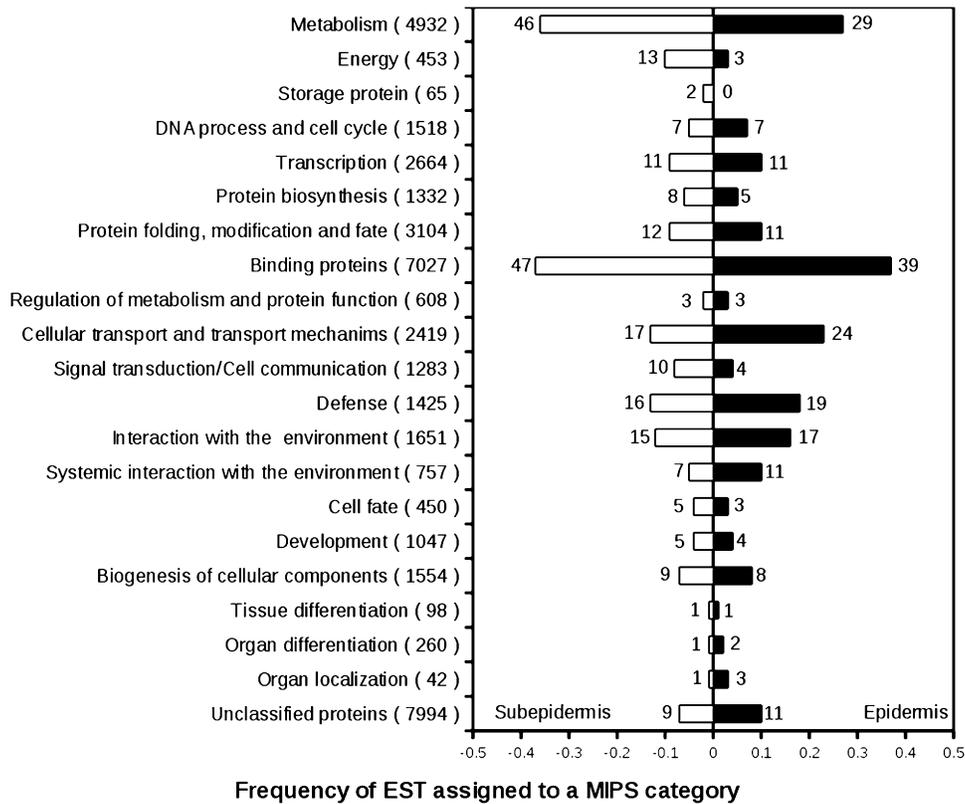
Fig. 4. Distribution of functional categories represented by the epidermis and subepidermis transcript samples from Citrus clementina fruit rind, based on the microarray data. The number of distinct unigenes from the epidermis (filled boxes) and subepidermis (open boxes) corresponding to each category is shown adjacent to the boxes, while the total number of unigenes from Arabidopsis assigned to that category is shown in parentheses, as assigned by MIPS (Munich Information Center for Protein Sequences, http://www.mips.gsf.de). The x axis shows the EST fraction preferentially expressed in epidermis cells and subepidermis cells, respectively.
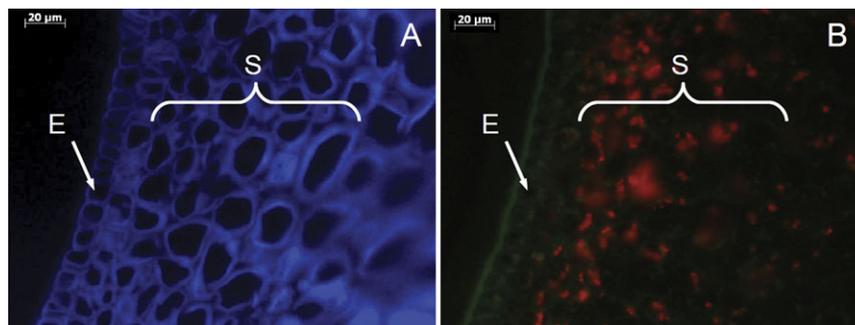

Fig. 5. Light microscopy image of *Citrus clementina* fruit rind. (A) Rind section stained with Calcofluor A as seen with a DAPI filter, showing the cell walls of the epidermis (E) and several inner layers of subepidermis (S). (B) The same tissue section as seen with a GFP filter showing the autofluorescence of the subepidermal chloroplasts (red) and the cuticle (thin green line) covering the epidermis.

no chloroplasts. It is suggested that there may be a high level of translocation of carbohydrates and energy-rich intermediates that are generated in the photosynthetically active subepidermal cells to the adjacent epidermal cells.

Another interesting observation was the high proportion of SP genes that were annotated as associated with the biosynthesis and restructuring of the primary cell wall, such as cellulose synthase, expansin, and pectin acetyl esterase. These genes were clearly expressed at substantially higher levels in the subepidermis and yet microscopic analysis

showed no apparent difference in wall morphology. Moreover, it is counterintuitive that the outer cell layer (the epidermis) would require less wall biosynthesis and remodelling than an inner layer, since basic physical principles dictate that the outer tissues of an expanding organ experience greater tensile stress than inner tissues (Kutschera and Niklas, 2007). However, it may either be that the cuticle plays a significant biomechanical role in the epidermal cells, and so the polysaccharide component is less essential, or that the outer epidermal layer does not provide the load-bearing

'skin' that constrains organ expansion, and that in this case this role is played by the subepidermis. Another explanation is that there are fundamental differences in the architecture and biomechanical properties between the cell walls of epidermal and subepidermal cells that are not reflected in wall thickness and the microscopy techniques used here.

A third important confirmation of the value of the cell-type dissection as a gene-enrichment strategy was the dramatic enrichment in EP genes associated with secretion, lipid metabolism, or more directly, cuticle biosynthesis, which specifically occurs in the epidermis. For example, large families of ABC transporters, that are often highly abundant in the epidermis, were identified (Panikashvili and Aharoni, 2008), cytochrome P450 proteins (Li *et al.*, 2010), *CER1, CER3, CER6*, and *CER8* (Aarts *et al.*, 1995; Lai *et al.*, 2007; Rowland *et al.*, 2007; Lu *et al.*, 2009), and lipid transfer proteins (Yeats and Rose, 2008). Interestingly, the most abundant EP gene is predicted to encode a member of the GDSL lipase protein family, the members of which have previously been proposed as contributing to cutin synthesis and turnover (Reina *et al.*, 2007; Pollard *et al.*, 2008). Moreover, several EP genes associated with flavonoid biosynthesis were identified, again suggesting that this biosynthetic pathway is epidermis specific. However, in addition to well-characterized biochemical pathways, the high proportion of EP genes that have no functional annotation was noted, which is interpreted to suggest that cell-type-specific metabolism may often be poorly understood in comparison with central metabolism, due the relative paucity of transcripts. Importantly, this would occur when the single cell layer of the epidermis is combined with the broader pool of fruit cell types, effectively diluting evidence of the related transcripts or proteins.

To conclude, it has been shown that the coupling of LMD with transcript profiling using DNA microarrays is a powerful platform to identify cell-type-specific transcripts and molecular pathways, and that such studies can provide new insights into aspects of not only cell-specific gene expression, but also tissue and organ physiology. It is further anticipated that with the emergence of next generation sequencing technologies, the utility of LMD will be further extended into species, such as citrus, that are not generally considered as models for molecular biology and functional genomics, but that represent excellent experimental systems for other aspects of plant biology.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. Primer sequences used for quantitative RT-PCR.

Supplementary Table S2. Epidermis predominant (EP) genes of *Citrus clementina* rind with array EST accession number, Citrus database contig, fold difference, and Citrus annotation as well as *Arabidopsis* orthologue best hit values, the presence of the SignalP motif, and other InterProScan domains.

Supplementary Table S3. Subepidermis predominant (SP) genes of *Citrus clementina* rind with array EST accession number, Citrus database contig, fold difference, and Citrus annotation as well as *Arabidopsis* orthologue best hit values, the presence of the SignalP motif and other InterProScan domains.

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