TECHNICAL ADVANCE

A new method for isolating large quantities of Arabidopsis trichomes for transcriptome, cell wall and other types of analyses

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Summary

A new procedure has been developed for the isolation of wild-type and mutant Arabidopsis trichomes. The isolated trichomes maintained enzymatic activity and were used for DNA, protein, and RNA isolation. The RNA was used to generate probes suitable for Affymetrix analysis. The validity of the Affymetrix results was confirmed by quantitative PCR analysis on a subset of genes that are preferentially expressed in trichomes or leaves. Sufficient quantities of trichomes were isolated to probe the biochemical nature of trichome cell walls. These analyses provide evidence for the presence of lignin in Arabidopsis trichome cell walls. The monosaccharide analysis and positive staining with ruthenium red indicates that the walls also contain a large portion of pectin. The 2.23-fold ratio of pectin-related sugars compared with potential cellulosic glucose suggests that the polysaccharides of the trichome cell walls are more like those of typical primary walls even though the wall becomes quite thick. Overall, these analyses open the door to using the Arabidopsis trichome cell wall as an excellent model to probe various questions concerning plant cell wall biosynthesis.

Keywords: trichome isolation, trichome cell wall, trichome gene expression, lignin, pectin.

Introduction

The development of Arabidopsis leaf trichomes has served as a model for the study of plant cell fate, pattern formation, and cell differentiation (Hulskamp, 2004; Marks et al., 1991; Szymanski et al., 2000). The attributes of trichome development in Arabidopsis as a model include accessibility (e.g. ease of visualization), relative simplicity (unicellular cell type), and dispensability (not required for laboratory growth).

Trichome development begins when single protodermal cells cease to divide and expand radially in the plane of the leaf surface. Thereafter, committed cells proceed through a series of readily identifiable stages of trichome differentiation. Stage one is characterized by the radial expansion in the plane of the leaf; stage two, expansion out of the plane of the leaf; stage three, development of branches; stage four, branch expansion; stage five, diffuse expansion; and stage six, cell wall maturation (Szymanski et al., 1998, 1999). Cell division does not occur during normal trichome differentiation, but trichome nuclear DNA does undergo several rounds of endoreduplication (Hulskamp et al., 1994).

Because trichomes are not required for plant growth in the laboratory, it has been possible to isolate many Arabidopsis mutants with trichome abnormalities. The onset of abnormal trichome development for many Arabidopsis trichome mutants begins at a discrete stage. For example, the abnormal development of glabra3-shapeshifter (gl3-sst)

trichomes is first apparent during stage two (Esch *et al.*, 2003). The *gl3-sst* trichomes over-expand during this stage. The trichomes on the *stichel* (*sti*) mutant lack branches, thus this mutant has an alteration in stage three when branches would normally initiate (Ilgenfritz *et al.*, 2003). The distorted class of mutants produce fairly normal trichomes up to stage five (Szymanski *et al.*, 1999). During stage five, rapid and general diffuse growth occurs throughout a wild-type trichome. However, the distorted mutants display altered expansion resulting in twisted and unevenly expanded trichomes. To better characterize wild-type and mutant trichomes, it would be desirable to isolate large quantities of trichomes suitable for biochemical and gene expression analyses. This report describes a new method for the isolation of Arabidopsis trichomes.

Two reports have described the isolation of mature Arabidopsis trichomes. In the first, Arabidopsis leaves were frozen in liquid nitrogen and then the trichomes were removed one at a time with forceps (Wienkoop *et al.*, 2004). An analysis of the proteins from 1000 to 2000 trichomes via nano-liquid chromatography–mass spectrometry (nano-LC/MS) allowed a limited number of trichome proteins to be identified. In another report, leaves were first fixed in a solution containing formaldehyde and glutaraldehyde, and then infiltrated with a solution containing EGTA (Zhang and Oppenheimer, 2004). Following incubation for 1–24 h the trichomes could be brushed off. Such trichomes have been used for cytoskeletal and morphometric analyses. However, it is likely that the fixation reduces the ability to isolate intact proteins and nucleic acids. This report describes a modified trichome isolation procedure that is faster and does not require the use of fixation. In addition, the procedure results in the isolation of much larger quantities of trichomes than previously described. The availability of larger quantities of a purified cell type has opened the door for many types of biochemical analyses that require larger amounts of tissue.

The purified trichomes have been used for protein, RNA, and DNA extractions. The extracted RNA has been used for the generation of aRNA biotin labeled probes for hybridization to Affymetrix chips, and a subset of the hybridization results have been verified by quantitative (q)PCR. In addition, a biochemical analysis of trichome cell walls has been performed. These latter studies provide data supporting the presence of lignin in Arabidopsis trichome cell walls. They also showed that the thick trichome walls have characters that blend those typically associated with primary and secondary walls since pectin is an abundant polysaccharide.

Results and discussion

To generate plant material for quick harvesting, Columbia wild-type plants were grown on flats of potting medium overlaid with perforated metal plates (Figure 1). Mature
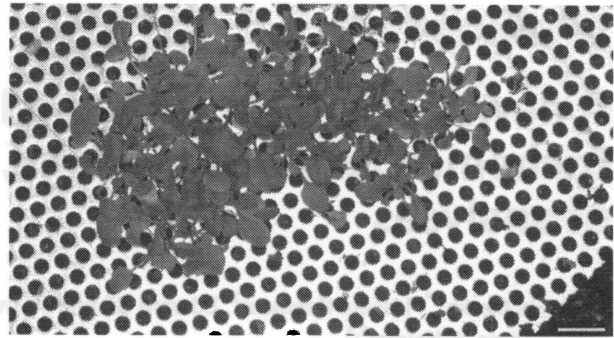
Figure 1. Columbia wild-type plants grown on a perforated metal plate. Image shows partially harvested flat of 28-day-old plants. Bar = 1 cm.

plants were rapidly harvested by shaving the plates with a razor blade. Several different methods were used to attempt to dislodge trichomes from the leaves of harvested material. The first method consisted of simply vortexing leaves in an isotonic PBS solution. This technique worked well for the isolation of glandular trichomes from such species as *Medicago truncatula* and *Cannabis sativa* (MDM, unpublished data). However, few trichomes were removed from Arabidopsis leaves. In a second attempt, small glass beads (60–80 µm) were added. Again, vortexing removed few trichomes. A third method made use of the previous finding that extended incubation with solutions containing EGTA could weaken the connection between trichomes and surrounding epidermal cells. This was presumably due to the chelation of Ca^{2+} and the subsequence weakening of the pectin component of the cell wall. However, short incubation in EGTA followed by vortexing failed to displace trichomes from the leaves. The addition of small glass beads and EGTA followed by vortexing greatly enhanced removal of trichomes from the leaves, as shown in Figure 2a. As described in Experimental procedures, these trichomes could be captured on 100-µm mesh filters. Large quantities of pure mature trichomes were easily and quickly isolated (Figure 2b,d). As judged by microscopic analysis, these trichomes have intact cytoplasm (e.g. arrows in Figure 2c). The unknown structures highlighted in Figure 2c are similar in size to plastids, which are very abundant in Arabidopsis trichomes (see Figure S1). Further analyses have shown that this technique could be used to isolate the trichomes from a range of different Arabidopsis trichome mutants (Figure 3a–c). These include *try*, *gl3-sst*, and *gl3-sst sim* trichomes, which have extra branches, exhibit variable shapes, or are composed of multicellular clusters, respectively (Esch *et al.*, 2003; Marks *et al.*, 2007; Schellmann *et al.*, 2002). This technique worked best on fully expanded leaves, as trichomes on younger leaves were rarely dislodged during the mixing (not shown).

Isolated trichomes were enzymatically active and were subjected to a variety of molecular procedures. For example,
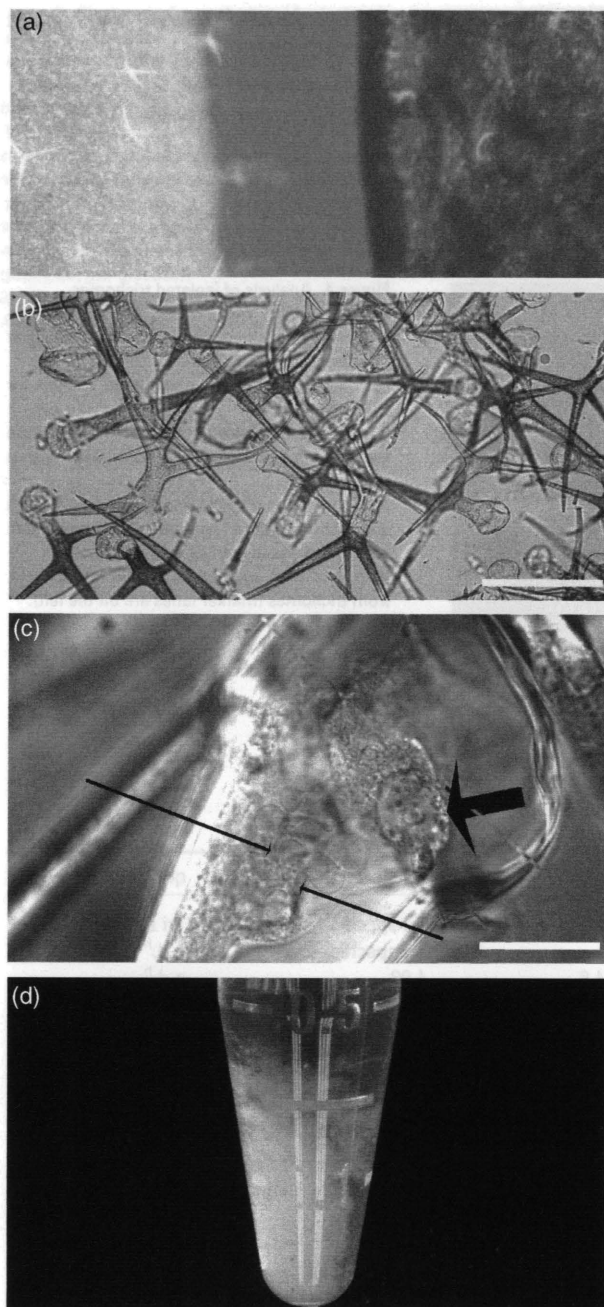
Figure 2. Isolation of mature trichomes from Arabidopsis leaves.
(a) Comparison of leaf epidermal surfaces before (left) and after (right) vortex treatment in solution containing EGTA and glass beads.
(b) Microscopic image of isolated trichomes.
(c) High magnification of an individual isolated trichome showing the presence of an intact cytoplasmic system.
(d) A 1.5-ml microfuge tube containing approximately 10,000 isolated trichomes.
The large arrow in (b) highlights the nucleus and the smaller arrows highlight unknown intact membrane bound structures. Bars in (b) 250 μm and (c) 25 μm.

the trichomes could be stained with 4′-6-diamidino-2-phenylindole (DAPI) to yield information on nuclear DNA content (Figure 4a), were assayed for GUS activity

(Figure 4b), or were visualized with fluorescence microscopy to study GFP localization (Figure 4c). In addition, the preparations were used for the isolation of trichome-expressed proteins, total RNA, or genomic DNA (Figure 4d–f).

RNA isolated from trichomes was useful for generating probes for transcriptome analyses using the Affymetrix ATH1 chip. In these analyses 200–500 ng of total RNA was used as template for labeling with the Ambion Biotin II Enhanced kit as described in Experimental procedures. In a preliminary analysis, probes were made from both isolated trichomes and from processed leaves from which the trichomes were derived. Previously, transcription factors such as *GL2, ETC1, TRY, CPC, TTG2*, which play roles in trichome formation, were shown to be preferentially expressed in trichomes (Esch *et al.*, 2004; Johnson *et al.*, 2002; Kirik *et al.*, 2004; Schellmann *et al.*, 2002; Szymanski *et al.*, 1998). The results from the Affymetrix analysis mirrored the previous findings (Table 1). Within the Affymetrix data, all these genes were shown to be highly expressed in trichomes, and only *TRY* expression was called present in the processed leaf transcriptome. The trichome-specific expression was not due to a faulty leaf sample, as other genes showed similar or higher expression in leaves compared with trichomes (Table 1). Quantitative PCR was used to further validate the Affymetrix results. In these analyses RNA isolated from different leaf and trichome samples was used to generate cDNAs for two-step qPCR. In all cases tested, the same trends in expression as found for the Affymetrix analysis were found for the qPCR analyses (Table 1). These results confirm that RNA isolated from trichomes as described can be used for Affymetrix transcriptome analyses. A spreadsheet containing the normalized results for trichome and processed leaf Affymetrix analyses is given in Supplementary File S1. While a more detailed analysis of the Arabidopsis trichome transcriptome involving additional replications will be presented elsewhere, an over-representation analysis (ORA) was performed to identify categories of genes that are more highly represented in trichomes than whole processed leaves. Several Gene Ontology (GO) biological process ontology groups having to do with cell wall biosynthesis were significantly over-represented ($P < 0.05$). These included groups of genes involved in carbohydrate biosynthesis ($P = 0.001$), cell wall precursor synthesis ($P = 0.0009$), and phenylpropanoid lignin biosynthesis ($P = 0.002$).

Trichomes have cell walls that are over 1 μm thick (Figure 5a). These cell walls should provide an excellent model system to study cell wall structure. Sufficient trichome material was isolated to conduct several cell wall analyses. The isolated trichomes were subjected to acid hydrolysis to release wall monosaccharides as described in Experimental procedures. The largest monosaccharide fractions were composed of glucose, galactose, and arabinose; followed by lesser quantities of mannose, rhamnose, xylose,

Figure 3. Trichomes isolated from different trichome mutants.
(a) *try-29760*.
(b) *gl3-sst*.
(c) *gl3-sst sim* double mutant.
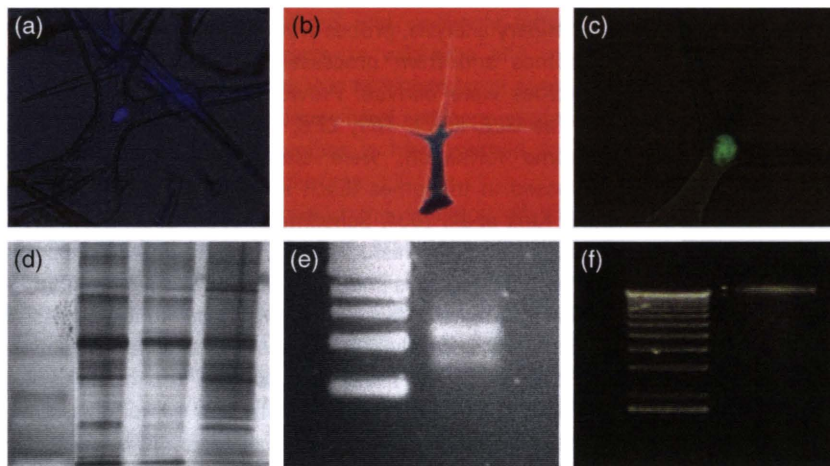
Figure 4. Analysis of isolated trichomes.
(a) Merged image of 4'-6-diamidino-2-phenylindole stained trichome showing nuclear fluorescence.
(b) Image of an isolated trichome stained for GUS activity.
(c) Merged fluorescence image of a trichome expressing a nuclear localized GFP fusion protein.
(d) Comparison of proteins isolated from leaves and trichomes (lane1, marker; lane 2, fresh leaf; lane 3, processed leaf; lane 4, trichome).
(e, f) Total RNA and genomic DNA, respectively, from trichomes (marker lanes are on the left).

Table 1 Comparison of gene expression in trichomes (Tri) and processed leaves

AGI	Name/function	Trichome[a]	Leaf[b]	Trichome/leaf[c]	qPCR trichome/leaf[d]
AT1G79840	*GL2*	9456	–[e]	Tri specific[f]	Tri specific[g]
AT1G01380	*ETC1*	3047	–[e]	Tri specific[f]	n.d.[h]
AT5G53200	*TRY*	969.7	194.6	4.98	n.d.[h]
AT2G46410	*CPC*	3186	–[e]	Tri specific[f]	222
AT2G37260	*TTG2*	1823	–[e]	Tri specific[f]	n.d.[h]
AT3G61260	DNA binding	4248	4252	1.00	0.84
AT5G02500	*HSC70-1*	1.39×10^4	1.36×10^4	1.02	0.38
AT3G59010	Pectinesterase	1.62×10^4	423.1	38.29	34
AT3G19710	*BCAT4*	124.6	7539	0.016	0.001

[a,b]Values from Affymetrix ATH1 chips hybridized to probes derived from either trichomes or leaves, respectively. Hybridization signals on the chips were normalized to 1000.
[c]Ratio of trichome over leaf using Affymetrix values.
[d]Ratio of trichome over leaf using qPCR as described in Experimental procedures.
[e]'–' indicates a probability of greater than 0.04 that the value was not above background.
[f]Expression is declared trichome specific if leaf value cannot be distinguished from background.
[g]*GL2* qPCR reaction using cDNA derived from leaf RNA required more than 37 cycles for detection (note: 26.23 ± 0.23 cycles were required for detection *GL2* cDNA derived from trichome RNA).
[h]Not done.

and fucose (Figure 5b). To test for the presence of lignin, the walls were subjected to thioacidolysis to release lignin subunits involved in arylglycerol-β-aryl (β-O-4) linkage as described in the Experimental procedures. This analysis revealed the presence of guaiacyl (G) units that are typically derived from lignin.

Histochemical staining was used to further characterize the walls (Figure 6). Interestingly, in all procedures used, the detached trichomes stained much better than intact trichomes on the leaves (compare Figure 6a and b, e and f, and g and h for intact versus detached trichomes for staining with Sudan black, ruthenium red and Maüle regent, respectively). These comparisons highlight the utility of using isolated trichomes for cell wall analysis. Consistent with the aliphatic outer layer frequently found in trichomes of diverse species (Peterson and Vermeer, 1984), Arabidopsis

Figure 5. Biochemical analysis of isolated trichome cell walls.
(a) Transmission electron microscope image of a cross-section through the cell wall of a trichome branch (bar = 0.5 µm).
(b) Quantification of trichome cell wall monosaccharides [left, histogram showing relative abundance of individual monosaccharides; right, gas chromatography (GC) trace of resolved monosaccharides (rha, rhamnose; fuc, fucose; ara, arabinose; xyl, xylose; man, mannose; gal, galactose; glc, glucose)].
(c) The GC-MS traces of thioacidolysis monomers (G, guaiacyl unit; S, syringyl unit).
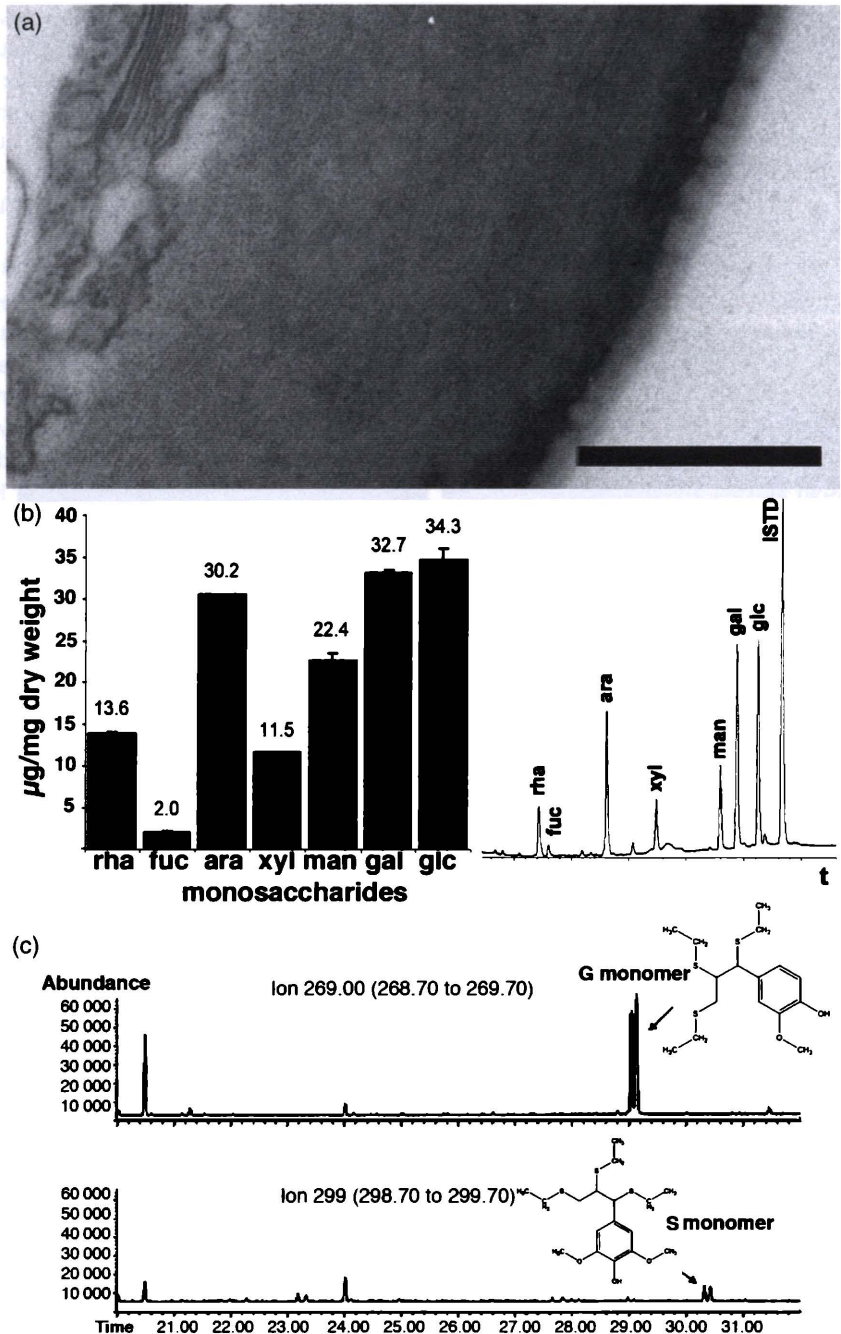
trichomes still attached to the leaf stained, albeit lightly, with lipophilic Sudan black B (Figure 6a). The outer layer was a barrier to the penetration of histological probes into the cell wall, for example Sudan black B stained isolated trichomes more intensely (Figure 6b). Similar to the Sudan black B result, three other probes yielded almost null reactions for trichomes attached to leaves, but strong reactions for isolated trichomes: (i) Tinopal LPW, a fluorescent brightener with affinity for cellulose (Taylor *et al.*, 1996) (Figure 6c,d); (ii) ruthenium red, a stain for pectin (Figure 6e,f); and (iii) the potassium permanganate–HCl (Maüle) reagent, which typi-

cally stains lignin (Figure 6g,h). Unstained trichomes had no autofluorescent signal (data not shown) under the same conditions used to record the Tinopal LPW fluorescence (Figure 6d). The Maüle reagent stained trichomes light brown (Figure 6h), whereas control gymnosperm (pine) lignin run in parallel stained dark brown (Figure 6i). Auto-fluorescence typical of lignin and other phenolics was observed in both isolated trichomes (data not shown) and those still attached to leaves (Figure 6j). In cases where branches of trichomes attached to leaves were broken so that stains could enter the lumen, signal intensity with
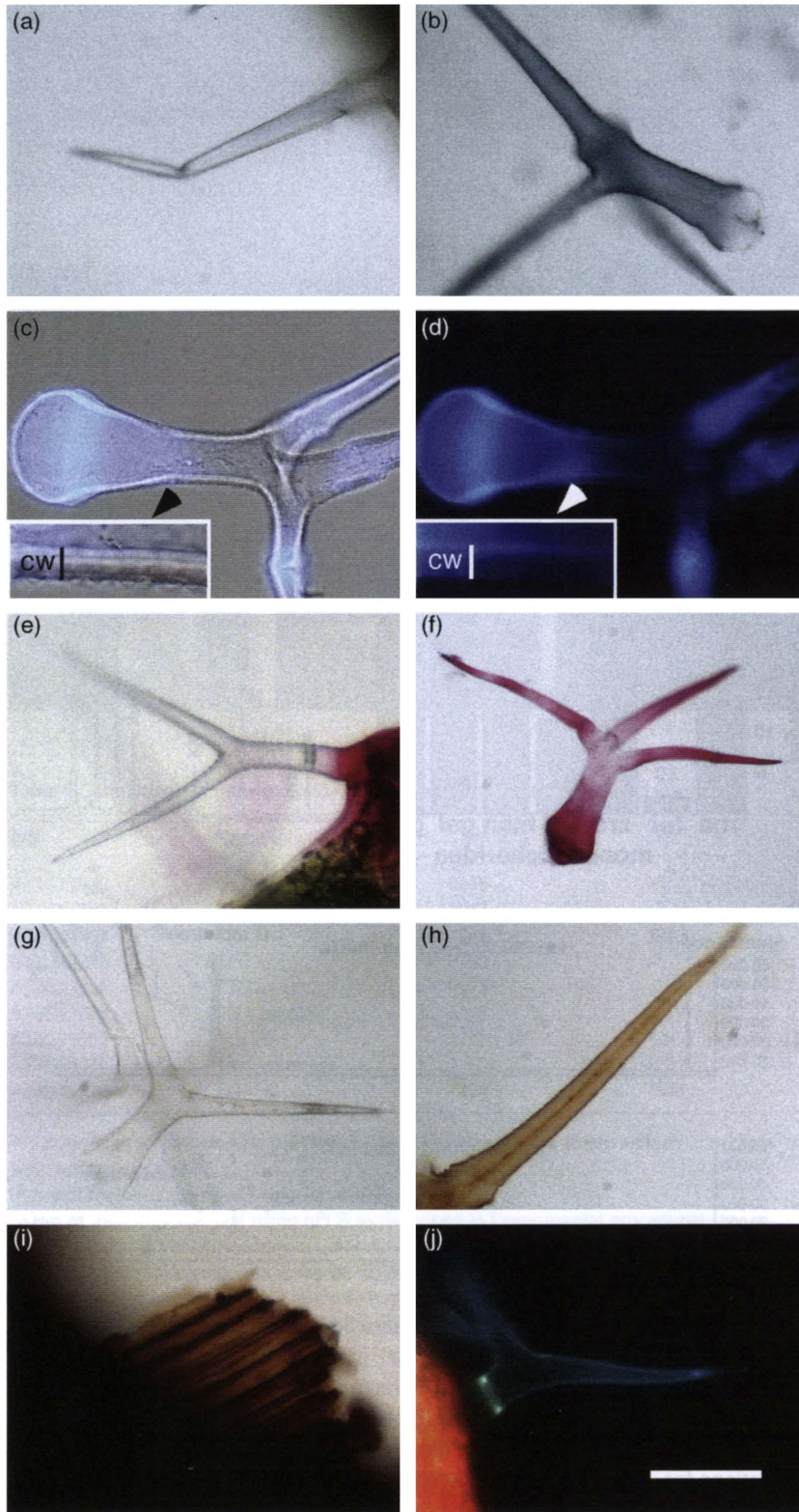
Figure 6. Light micrographs of stained Arabidopsis trichomes, either isolated or attached to a leaf.

(a, b) Sudan black B stained the lipids of attached trichomes (a) lightly and those in isolated trichomes more strongly (b).

(c, d) Tinopal LPW, a fluorescent probe for cellulose, stained only isolated trichomes. No fluorescent signal was detected in attached trichomes photographed under the same conditions (data not shown). Part (c) shows simultaneous fluorescent and brightfield imaging, whereas (d) shows only Tinopal LPW fluorescence from the same trichome. Insets in (c) and (d) are higher-magnification views of the cell wall area indicated by the arrowheads, with cell wall (cw) width indicated by the vertical line in each case. Comparison of (c) and (d) shows that in the stalk of the trichomes, Tinopal LPW only reacted with the innermost layer of the thick cell wall. Intense spots of fluorescence in branches correspond to bends (created during sample processing) where wall structure was probably disrupted, allowing more access of the fluorochrome to internal cellulose.

(e, f) Ruthenium red, a stain for pectin, was null on attached trichomes (e), but reacted strongly with isolated trichomes (f). The micrograph in (e) also illustrates that ruthenium red did not react with an intact leaf through its cuticle (lower green area) but did penetrate and stain the cut edge of a leaf (right side) as well as the basal cell of a nearby trichome.

(g, h) The Maüle reaction, which stains guaiacyl units brown, was null on attached trichomes (g) and positive on detached trichomes (h).

(i) The Maüle reaction stained pine wood dark brown.

(j) Autofluorescence of an attached trichome; similar results were observed for isolated trichomes (data not shown). The bar in (j) = 100 μm is for all large micrographs in (a – j). For the insets in (c) and (d), the bar in (j) corresponds to 31.5 μm.

Tinopal LPW and ruthenium red was similar to that with isolated trichomes (data not shown).

The transmission electron microscope (TEM) image of the trichome wall (Figure 5a), with its thin outer electron trans-

lucent layer and dark-staining layer underneath, resembles walls of regular epidermal cells that have a waxy outer layer underlain by a cuticulated cell wall. In this specialized type of wall, cutin is typically interspersed with pectin and cellulose

(Fahn, 1990). The more intense Sudan black B staining of isolated trichomes, in which the stain could move through the cell interior, is consistent with cutin existing within the cell wall. The binding of Tinopal LPW selectively to the inner surface of the thick wall in the stalk of isolated trichomes is also consistent with the binding sites of cellulose being covered by other molecules in the bulk of the wall, but not the inner layer (Figure 6c,d). Logically, the outer layer of the trichome must be highly hydrophobic in order to maintain the overall epidermal water barrier. However, both cuticulated and lignified cell walls (see below) stain densely black in other plant samples prepared for TEM and either cutin or lignin could block the staining of cellulose. Therefore, further work with more specific probes will be required to determine the exact mixture of hydrophobic molecules that are intercalated into the trichome cell wall.

The staining of isolated trichome walls with Tinopal LPW is consistent with a cellulosic component, as characterized before by the reduction of birefringence (in the polarizing microscope) and acetic/nitric-insoluble cellulose in the *tbr* mutant (Potikha and Delmer, 1995) and with the substantial levels of glucose (34.3 µg mg^{-1}) in the total trichome cell wall monosaccharides that were assayed as alditol acetate derivatives after H$_2$SO$_4$ hydrolysis (Blackeney *et al.*, 1983). The large cumulative proportion of rhamnose, arabinose, and galactose (76.5 µg mg^{-1}), as well as positive staining with ruthenium red, is consistent with the presence of a large amount of pectin. Although part of the glucose may be in non-cellulosic polysaccharides such as xyloglucan, the 2.23-fold ratio of pectin-related sugars compared with potential cellulosic glucose suggests that the trichome wall is more like typical primary walls even though it becomes quite thick. Accounting for the other monosaccharides, xylose (11.5 µg mg^{-1}) may be a component of xyloglucan or xylan (although this typically occurs in classical cellulose-rich, pectin-depleted secondary walls such as those in xylem) and fucose (2.0 µg mg^{-1}) may be a minor component of pectin and xyloglucan. The mannose (22.4 µg mg^{-1}) may be found in cell wall mannans, which are known to occur as matrix components in the thickened outer walls of regular epidermal cells of Arabidopsis stems and leaves (Handford *et al.*, 2003).

Although soluble phenolics as deterrents of herbivory are commonly found in trichomes, the existence of lignin in the cell walls has not been documented before (Peterson and Vermeer, 1984). Our data support the existence of lignin in trichome cell walls, which is consistent with their high stiffness and strength as part of their defensive roles. Results of thioacidolysis showed the predominance of guaiacyl (G) units that are typically found in lignin, which corresponded with a brown chromophore arising in trichome walls from the potassium permanganate–HCl (Maüle) test (Dean, 1997). Gymnosperm (pine) lignin, with abundant G-lignin, also stained brown in a control reaction. Although syringyl

(S)-units would stain red in this reaction, thioacidolysis indicated that they are the minor component of lignin in the trichome cell walls. In contrast, the phloroglucinol–HCl test (the Weisner reaction) producing a red reaction on wood pulp fiber run in parallel was null on both isolated and intact trichomes (data not shown). The red chromophore typically arises from the acid-catalyzed condensation of phloroglucinol with coniferaldehyde (free aldehyde) units of lignin to yield cinnamaldehydes (Dean, 1997). However, examples of angiosperm (transgenic tobacco) lignin without phloroglucinol-positive free aldehydes are known (Franke *et al.*, 2000), and it is also possible that free aldehydes in trichome walls are blocked by other molecules. Like lignin, suberin also contains hydroxycinnamic acids and their derivatives and is electron dense after fixation and staining for TEM. However, suberin is usually specifically located at the plasma membrane/cell wall interface (Nawrath, 2002). In contrast, the trichome walls have their maximum electron density toward the outer surface, which makes it less likely that the positive Maüle stain in trichomes arose from any guaiacyl terminal units that might exist in suberin.

In conclusion, a robust procedure for isolating Arabidopsis trichomes suitable for a wide range of biochemical and molecular analyses has been described. This procedure should facilitate the use of Arabidopsis trichomes in the study of many topics including the epigenetic modifications of genomic DNA, transcriptome analyses, enzyme activity assays, metabolite analyses, cell wall analyses, etc. Of particular note, the cell walls of the Arabidopsis trichome provide a good model system for the analysis of wall structure and biochemistry. Given that plants do not require trichomes, it should be possible to genetically engineer and manipulate trichome cell walls in ways not possible for whole plants.

Experimental procedures

Growth of plants

To minimize contaminating potting medium and to speed the isolation of plant material, Arabidopsis plants were grown on flats containing Sunshine LP5 potting medium (Sun Gro Horticulture, http://www.sungro.com/) overlaid with perforated metal plates (Figure 1). Plants were grown under continuous illumination at 22°C. After approximately 4 weeks, seedlings were harvested by shaving the plants off the metal plates with razor blades. Seedlings were rinsed twice in tap water to remove extraneous debris. Columbia wild-type plants were used for all analyses.

Trichome isolation

Seedlings were stuffed (approximately 1.5 g per tube) into 50-ml test tubes (Greiner Bio-One, http://www.greinerbioone.com/) containing approximately 50 mg of 60/80 µm glass beads (Alltech, part number 5420, http://www.discoverysciences.com/) and 15 ml of a solution containing 50 mM ethylene glycol-bis

(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA pH 7.5 with KOH; Sigma-Aldrich, http://www.sigma-aldrich.com) and 1× modified phosphate-buffered saline (PBS). The PBS solution was essentially made as described in Sambrook *et al.* (1989) with the exception that all potassium salts were used. Tubes containing the seedlings were mixed at maximum speed (four cycles of 30 sec on and 30 sec rest on ice) on a Genie 2 vortex (Scientific Industries, http://www.scientificindustries.com/). It was important not to overload the seedlings in the tubes to the point that they could not freely rotate during mixing. Up to 12 tubes of seedlings were processed at the same time. The processed seedlings were collected in a 500-ml beaker and the solution was strained through four layers of screen door mesh (Home Depot, http://www.homedepot.com/) into a flask. The seedlings in the beaker were rinsed several times with the PBS solution (no EGTA) to free trichomes trapped in the plant material. The rinse solutions were filtered and combined in the flask with the original filtrate. The resulting solution was sieved through a 100-µm cell strainer (Falcon-Becton Dickinson, http;//http://www.bdbiosciences.com/). Trichomes ensnared on the mesh were rinsed with several milliliters of PBS, and then the cell strainer was inverted into a small Petri dish. Approximately 10 ml of PBS solution was used to dislodge the trichomes from the filter. Any remaining plant debris that co-purified with the trichomes was removed with fine forceps. The solution containing the trichomes was transferred to a 15-ml centrifuge tube and spun at 150 *g* for 1.5 min. The supernatant was carefully removed, leaving the trichomes ready for downstream processing. Five grams of seedlings was sufficient to isolate approximately 15 000 trichomes, as quantified with a hemocytometer.

Staining and imaging

Trichomes were directly assayed for GUS activity, DAPI stained for DNA content, or examined for GFP fluorescence as previously described (Marks *et al.*, 2007).

RNA, DNA, and protein isolation

Trichomes destined for RNA isolation were placed in a solution of RNAlater (Ambion, http://www.ambion.com/) and subjected to a vacuum for 5–10 min for infiltration. Trichomes were stored in RNAlater for up to a month or more at 4°C. For RNA isolation, up to 100 mg (approximate wet weight) of trichomes was moved to a 1.5-ml microfuge tube and centrifuged for 30 sec at 350 *g*. Residual RNAlater was then removed. RNA was isolated from the trichome pellet using the Plant RNeasy kit (Qiagen, http://www.qiagen.com/) with one modification. The trichome pellet was resuspended in 100 µl of supplied RLT buffer and then frozen solid in liquid nitrogen. As the pellet thawed, it was ground to a fine paste using a small pestle. An additional 350 µl of RLT was added and then the RNA was isolated following the kit's instructions. After isolation the RNA was subjected to DNase treatment using TurboDNAse (Ambion) and then concentrated using a RNA MinElute spin column (Qiagen). RNA was eluted in a small volume and stored at −80°C.

For protein isolation, approximately 50–100 mg (wet weight) of trichomes was frozen in liquid nitrogen in a 1.5-ml microfuge tube and ground to a fine powder with a pestle. Before the samples thawed, 50–100 µl of 2× Leammli SDS-PAGE buffer (Weigel and Glazebrook, 2002) was added and the trichomes were subjected to further grinding. The samples were boiled for 2 min and centrifuged at maximum speed in a microfuge for 5 min. Thirty microliters of the supernatants were resolved on a precast BioRad 5–20% SDS polyacrylamide gel (http://www.bio-rad.com/) and the proteins were visualized by silver staining. DNA was isolated using the Master-Pure Plant Leaf DNA Purification Kit (Epicentre Biotechnologies, http://www.epibio.com/).

Monosaccharide analysis

Isolated trichomes were extracted twice in 70% ethanol at 70°C and once in acetone. Trichomes were dried in a desiccator and inositol was added as an internal standard to 0.5–1 mg material. The trichomes were subjected to hydrolysis and acetylation as previously described (Blakeney *et al.*, 1983). The acetylated monosaccharides were extracted with methylene chloride and directly subjected to gas chromatography (GC) analysis using an Agilent gas chromatograph (6890N; Agilent, http://www.agilent.com/chem/GC) equipped with a Supelco SP-2330 capillary column (Sigma-Aldrich) as described in Bauer *et al.* (2006). Individual acetylated monosaccharide standards plus inositol were run for comparison.

Lignin analysis

Thioacidolysis was used to study the lignin composition of isolated trichomes as previously described (Chen *et al.*, 2006; Lapierre *et al.*, 1986, 1995). Briefly, thioacidolysis reagent was added to 1 mg dried trichomes. Samples were heated for 4 h at 80°C. Docosane was added as an internal standard, followed by water and a solution of saturated $NaHCO_3$ (final pH 3–4). Samples were extracted three times with methylene chloride and the combined extractions were dried under N_2. Samples were resuspended in pyridine and silylated with *N*-methyl-*N*-trimethylsilytrifluoroacetamide to increase the volatility of the thioethylated monomers. The resulting suspensions were subjected to GC-MS. The main thioacidolysis monomer peaks were obtained from the total ion chromatogram using a *m/z* of 239 for the ethanethiol derivative of *p*-hydroxyphenyl (H) units, 269 for guaiacyl (G) units and 299 for (S) syringyl units.

Affymetrix analysis

The MesageAmp II-Biotin Enchanced kit (Ambion) was used following kit directions to convert total trichome or leaf RNA into biotin-labeled aRNAs. During this procedure a single round of T7 polymerase-driven amplification was used to generate the biotin-labeled aRNA. As little as 300 ng was sufficient to generate over 15 µg of probe. For hybridization, 15 µg of aRNA was fragmented using the supplied fragmentation buffer and the resulting products were sent to the University of Minnesota BioMedical Genomics Center for hybridization to Affymetrix ATH1 Genchips (Affymetrix, http://www.affymetrix.com/). The resulting data files were normalized and analyzed using Expressionist software (Genedata, http://www.genedata.com/products/expressionist/).

qPCR

Quantitative PCR analyses of select genes were performed using the Lightcycler (Roche, http://www.roche.com/) as previously described in detail (Marks *et al.*, 2007). Briefly, shoot cDNA was used as a template in a conventional PCR reaction with the primers shown below to generate DNA fragments to be used as standards. Standards were gel purified and serially diluted. Diluted standards and equivalent amounts of cDNA derived from either processed leaves or isolated trichomes were amplified. Relative levels of leaf and trichome expression were calculated with Lightcycler Software

using the values derived from the reactions containing the diluted standard DNA fragments. All reactions were performed in duplicate along with a control without DNA.

Shown below are the genes that were analyzed by qPCR along with the sequence of the primers that were used and the size of products:

AT3G61260 DNA-binding
293 bp product
For GATGACTCCAAAGCCCTTACT
Rev CTCTCTGCATACTCTGCTTTCT
AT5G02500 *HSC70-1*
305 bp
For AACCCCGTTAACACCGTTTTC
Rev CAGCATCCTTTGTAGCCTGA
AT3G59010 pectinesterase
341 bp
For AGTAGAAGAGCTGAGACCTC
Rev CTACAGCTTGCTCCGAGTTT
AT3G19710 *BCAT4*
283 bp
For AGTGGAACTGGTGGTGTGAA
Rev CCACTAGAGGAATCGTACGT
AT2G46410Q *CPC*
211 bp
For AGGCTTCTTGTTCCGAAGAGG
Rev AAGTCTCTTCGTCTGTTGGCAA
At1g79840Q *GL2*
442 bp
For CGAGAACTCAGGACCCACGAGATC
Rev GGAGTTTTCGAGGTGGAGATCATCGG

Histochemical assays

Trichomes, both isolated and attached to leaves, were processed in parallel. Procedures for staining were: (i) Maüle reaction and ruthenium red according to (Chaffey, 2002); (ii) 10 min in 1% (w/v) phloroglucinol in a 55% (v/v) dilution of concentrated HCl, followed by mounting in distilled H_2O and immediate observation; (iii) 10 min in 0.1% (w/v; saturated) Sudan black B, followed by a brief wash in 50% ethanol, then mounting in water; (iv) 5 min in 0.005% (w/v) Tinopal LPW® (a gift from Ciba Geigy, now Ciba, http://www.cibasc.com; CI no. 40622, equivalent to Calcofluor®) in PBS and mounting in PBS. Micrographs were captured with an Olympus BH-2 microscope (http://www.olympusmicro.com) and a Q-5 cooled digital camera (http://www.qimaging.com). Dyes were photographed with brightfield optics (Koehler illumination and condenser aperture fully open so that contrast arose primarily from the stains). Tinopal LPW fluorescence was captured with modified ultraviolet excitation (UG-1 with an additional 20 nm narrow band pass at 365 nm), and autofluorescence was captured with violet (V; BP405) excitation (http://www.olympusmicro.com). Digital images were optimized by histogram (Levels) adjustment in Adobe Photoshop, with identical processing of any two images in which staining intensity was compared.

TEM analysis

Young leaves were harvested from 20-day-old seedlings and fixed in Karnovsky's fixative (1% paraformaldehyde + 0.1% glutaraldehyde). After several rinses in PBS buffer, tissue was refixed with 1% osmium tetroxide. Tissue was dehydrated by a series of incubations

in solutions containing increasing concentrations of ethanol. Ethanol was replaced with LR white for embedding. All incubations were performed using the PELCO 3441 Tissue Microwave Processing System (Ted Pella Inc., http://www.tedpella.com/). Incubation times and other details are available on request. Blocks containing tissue were trimmed and sectioned in an RMC MT-7000 ultramicrotome (RMC Inc., http://www.rmcproducts.com/). Sections of 85 nm were viewed using a Phillips CM12 transmission electron microscope.

Over representation analysis (ORA)

The 5000 most highly expressed genes in the trichome and processed leaf datasets were compared using the ORA tool located at http://mapman.mpimp-golm.mpg.de/general/ora/ora.shtml and described in Usadel *et al.* (2006).

Supporting Information

Additional Supporting Information may be found in the online version of this article:
File S1. An Excel spreadsheet containing the normalized results from the hybridization of biotin-labeled probes (derived from total RNA isolated from either trichomes or processed leaves) to the Affymetrix ATH1 chip.
Figure S1. Maximum projection of a confocal image of a mature trichome expressing a YFP-plastid linked marker.
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