Transpiration from Tomato Fruit Occurs Primarily via Trichome-Associated Transcuticular Polar Pores

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Nonstomatal water loss by transpiration through the hydrophobic cuticle is ubiquitous in land plants, but the pathways along which this occurs have not been identified. Tomato (Solanum lycopersicum) provides an excellent system in which to study this phenomenon, as its fruit are astomatous and a major target for desiccation resistance to enhance shelf life. We screened a tomato core collection of 398 accessions from around the world and selected seven cultivars that collectively exhibited the lowest and highest degrees of transpirational water loss for a more detailed study. The transpirational differences between these lines reflected the permeances of their isolated cuticles, but this did not correlate with various measures of cuticle abundance or composition. Rather, we found that fruit cuticle permeance has a strong dependence on the abundance of microscopic polar pores. We further observed that these transcuticular pores are associated with trichomes and are exposed when the trichomes are dislodged, revealing a previously unreported link between fruit trichome density and transpirational water loss. During postharvest storage, limited self-sealing of the pores was detected for certain cultivars, in contrast with the stem scar, which healed relatively rapidly. The abundance of trichome-associated pores, together with their self-sealing capacity, presents a promising target for breeding or engineering efforts to reduce fruit transpirational water loss.

In later-diverging land plants, water is lost primarily by transpiration through stomata. However, when tissues begin to dry, the stomata close, restricting transpiration through this low-resistance route (Saliendra et al., 1995; Brodribb and Holbrook, 2003). Indeed, under such conditions, stomata contribute very little to the overall rate of water loss (Burghardt and Riederer, 2003; Santrucek et al., 2004) and transpiration mostly occurs directly from the apoplast of epidermal cells. The hydrophobic cuticle that coats the epidermis of aerial organs provides the key barrier against this flux; however, despite extensive research, the key factors that determine the permeances of cuticles, which vary by over 500-fold across plant species (Riederer and Schreiber, 2001), have not been fully resolved.

Answering this question is complicated by the compositional and structural complexity of cuticles, which consist of a lipidic polyester, termed cutin, along with polysaccharides and various soluble compounds referred to collectively as waxes (Riederer and Muller, 2006; Schreiber, 2010; Yeats and Rose, 2013). Furthermore, each of these constituents is compositionally diverse, with the types and amounts varying between species and even between organs of a single individual (Riederer and Muller, 2006; Schreiber, 2010; Yeats and Rose, 2013). However, cuticular waxes are believed to be critical for restricting transpiration, as various studies have found that extraction of the wax component increases permeance by 100- to 2,000-fold (Schreiber and Schönherr, 2009; Schreiber, 2010). In contrast, the >90% reduction in cutin levels in fruit of the cutin deficient2 (cd2) and cd3 mutants of tomato (Solanum lycopersicum) were reported to have a minimal effect on water loss (Isaacs et al., 2009). Despite the substantial effect of waxes, there does not appear to be a correlation between total wax levels and cuticular permeance across, or within, species (Jordan et al., 1984; Schreiber and Riederer, 1996; Riederer and Schreiber, 2001; Parsons et al., 2013). Indeed, a single monolayer of wax could theoretically account for the entire resistance of a cuticle to water diffusion (Schreiber and Schönherr, 2009).
The wax compositional profile, rather than amount, is likely to be the key determinant of permeance.

Triterpenoids, a common class of cyclic waxes, do not appear to contribute to transpirational resistance (Grncarevic and Radler, 1967), and several studies have found that their abundance relative to aliphatic wax components correlates with increased permeability (Vogg et al., 2004; Leide et al., 2011; Buschhaus and Jetter, 2012; Parsons et al., 2013; Jetter and Riederer, 2016). Thus, the ratio of aliphatic compounds to triterpenoids has been proposed as a key determinant of differences in permeability of up to 8-fold in the most extreme case (Leide et al., 2011). However, importantly, this only accounts for a small amount of the total variation in permeance observed between cuticles from different sources. An alternative hypothesis is that there exist polar pores, possibly created by the presence of polysaccharides that span the cuticle and provide a lower resistance route for water movement and largely dictate water flux (Schreiber, 2005). Diffusion experiments with isolated cuticles have offered support for this idea (Schönherr, 1976, 2006; Popp et al., 2005), but the presence of such pores has yet to be demonstrated. If they indeed exist, variation in their abundance or size might result in substantial differences in cuticular transpiration rates.

Tomato fruit represents a useful model system in which to study the relationships between cuticle structure, composition, and properties, as they are amenable and can be easily isolated, allowing direct measurement of permeance and biomechanical characteristics (Martin and Rose, 2014). Notably, while there is considerable variability in the amount and composition of waxes among tomato cultivars (Bauer et al., 2004), studies to date of tomato fruit cuticles have largely focused on specific genetic variants or mutants, with little utilization of varietal diversity. A recent study has shown that even among just three varieties, there is significant variation in fruit transpiration rate and cuticle permeability (Romero and Rose, 2019). In this study, we used a germplasm diversity panel of tomato accessions, similar to the collection described by Lin et al. (2014), and assessed the range of fruit cuticular permeance. Specifically, we investigated water loss rates from harvested mature green (MG)-stage fruits and asked whether variation in cuticular permeability can be explained by wax composition or whether other cuticle properties or features play an important role.

RESULTS

Fruit Water Loss Rates

We measured the rate of transpirational water loss from detached MG fruits from 398 tomato accessions, including 307 accessions of *S. lycopersicum* (cultivated tomato), 42 of *Solanum pimpinellifolium* (the closest wild relative of cultivated tomato), and 51 of *S. lycopersicum cv cerasiforme* (small-fruited varieties that are genetically intermediate between *S. lycopersicum* and *S. pimpinellifolium* wild tomato). We observed substantial variability in transpiration rates across the panel (Fig. 1), particularly among the *S. lycopersicum* lines, which ranged from 1.4 to 6.7 mg cm⁻² d⁻¹, with a median value of 2.4 mg cm⁻² d⁻¹. The *S. lycopersicum cv cerasiforme* and *S. pimpinellifolium* fruits showed narrower ranges, with the former tending toward higher rates of water loss (median of 2.8 mg cm⁻² d⁻¹) and the latter showing lower rates (median of 2 mg cm⁻² d⁻¹). *S. lycopersicum* varieties from the extreme ends of the range were selected for further analysis.

Four low-water-loss lines and five high-water-loss lines were regrown under controlled greenhouse conditions to confirm the phenotypes observed in the diversity panel screen. Of the four low-water-loss lines, three again showed low transpiration rates (lines 10022, 9959, and 10177), while the fourth (line 10241) had an intermediate rate (Fig. 2A). Only three of the five high-water-loss lines reproducibly showed this phenotype: 10062, 10077, and 9999 (Fig. 2A), and the two other lines were not considered further. Thus, a total of seven lines, representing seven distinct cultivars, were carried forward for further analysis. These were categorized as small-fruited varieties (10022, 9959, 10062, and 10077) and medium-fruited varieties (10177, 10241, and 9999), and these lines are hereafter compared within their size class, to avoid any possible influence of fruit size on any of the phenotypes.

Cuticular Permeability

To test whether the differences in the whole-fruit transpiration rates are dictated by differences in cuticle permeance, we measured water loss through isolated MG-stage cuticles, using a transpiration chamber system (Supplemental Fig. S1). We calculated the permeance of the cuticles from the different cultivars (Fig. 2B), taking the humidity difference across the cuticle membrane as the driving force for the flux of water. These values ranged from 3.4 × 10⁻⁵ to 3.6 × 10⁻⁵ m s⁻¹ for the low-water-loss varieties and 4.8 × 10⁻⁵ to 7.1 × 10⁻⁵ m s⁻¹ in the high-water-loss lines. These relative values among the varieties were consistent with the whole-fruit transpiration rates, except that the cuticular permeance of line 10241 was more similar to that of the other low-water-loss varieties.

Whole-Cuticle and Cutin Analysis

Light microscopic imaging of outer pericarp sections from MG-stage fruit, which had been stained with Oil Red O to highlight the cuticle, did not show any obvious structural defects in any of the lines. However, we observed anatomical variability between the cultivars, with differences in epidermal cell shape and subepidermal cuticle deposition (Supplemental Fig. S2). The epidermal cells in lines 10022, 10062, 10077, and 9999...
had an oval shape, while those in lines 10241 and 10177 were rectangular, and the line 9959 epidermis consisted of triangular cells. In addition, lines 10022 and 9959 were unique in containing substantial cuticle deposition in subepidermal cell layers.

We next tested for correlations between cuticle permeance, cuticle abundance, and cuticle composition. The amount of cuticle covering a given area of the epidermis was measured in four different ways: (1) total cuticle mass per unit area of isolated cuticles was determined gravimetrically (Fig. 3A); (2) cutin abundance was determined by quantifying levels of cutin monomers following cutin depolymerization, using gas chromatography (GC) analysis (Fig. 3B); (3) cuticle thickness was measured from light microscopy images, based on the distance from the cuticle outer surface to the edge of the epidermal cell (over-epidermal cell); and (4) thickness was similarly measured from the outer surface to the point of its deepest penetration into the tissue (penetration depth; Fig. 3, C and D). Cuticle mass, cutin abundance, and cuticle penetration depth showed a consistent pattern across the seven varieties, with line 10022 having the highest values and line 10241 having the lowest. Both of these varieties have low fruit cuticular transpiration rates. With regard to the over-epidermal thickness, line 10241 again had the lowest values; however, by this measure, the thickest was line 9999, which is a high-water-loss line.

The composition of the cutin showed little variation between the lines, with one exception being a deficit of hydroxylated 16-carbon monomers and relative increase in unidentified compounds in line 9959 (Supplemental Fig. S3). None of the patterns in these traits, relating to the whole cuticle or cutin, correlated with the cuticle permeance values.

**Cuticular Wax Analysis**

We next measured the amount and composition of cuticular waxes on MG fruits by GC analysis (Fig. 4; Supplemental Fig. S4) to determine whether these factors might explain the variability in water loss rates. The amounts of total wax mirrored the pattern observed for the whole-cuticle and cutin measurements and thus did not correlate with permeance. We detected substantial differences in wax composition among the lines (Supplemental Fig. S4), which is reflected in the aggregate amounts of alkanes and triterpenoids (Fig. 4). The high levels of wax in the low-water-loss lines 10022 and 9959 reflect exceptionally high amounts of triterpenoids,
which were present at average, or below-average, levels in the other two low-water-loss lines. The amounts of alkanes were fairly consistent across the cultivars, with the exception of line 10177, which had particularly high alkane levels. Thus, neither the total wax amount nor the amounts of individual waxes showed a correlation with permeance.

Permeable Pores

The surprising lack of a relationship between the measured cuticle compositional parameters and the observed variation in cuticular transpiration rates suggested that some other factor is primarily responsible for water loss. While checking the integrity of whole fruits or cuticle membranes using the hydrophilic dye Toluidine Blue (TB), we noticed staining of numerous barely visible puncta, distributed randomly and relatively evenly over the fruit surface (Fig. 5A; Supplemental Fig. S5). When fruit were stained for an extended period of 3 d, the dye could be seen permeating through the underlying tissue surrounding the point of penetration (Fig. 5A, inset). The binding of the stain at the surface and its diffusion through to the underlying tissue suggested that the spots were hydrophilic and that they extended through the cuticle to the underlying apoplast. We counted the numbers of blue spots per unit area of isolated fruit cuticles from the seven cultivars (Supplemental Fig. S5) and compared the average spot densities with the average cuticular permeance values. The two parameters showed a strong positive correlation (Fig. 5B; \( P = 9.1 \times 10^{-5} \)), with the spot densities able to explain almost all of the observed variation in permeance values (\( R^2 = 0.96 \)).

This relationship was further tested by analyzing the variation of both traits among individual cuticle samples from ripe fruit of one reference cultivar, M82. To account for possible differences in water conductivity among the puncta, their cumulative effect was quantified in terms of the total area stained with TB after a 2-d incubation. A strong correlation was found between the cuticular permeances and the TB-stained area, implying a significant effect of the TB-penetrable puncta on fruit transpiration rate (Fig. 6; \( P = 1.63 \times 10^{-8} \)). We inferred from this result that the spots correspond to hydrophilic pores that provide a low-resistance route for water flux. The average permeance of a pore-free cv M82 cuticle

Figure 3. Amount of cuticle from MG fruits, quantified by four methods. A, Mass per unit area, determined gravimetrically from isolated cuticles (\( n = 8 \)). B, Total amount of cutin, measured by GC (\( n = 3 \)). C, Two measures of cuticle thickness, based on light microscopy images of Oil Red O-stained pericarp sections, as shown in D. \( n = 6 \), with each replicate representing the mean of nine to 12 measurements from one fruit. Mean values of biological replicates \( \pm \) se are presented for each section.

Figure 4. Total amount of cuticular wax extracted from MG fruits, as well as the amounts that were composed of alkanes or triterpenoids. Abundances were measured using GC (\( n = 5 \)). Mean values \( \pm \) se are shown.
was estimated as $1.49 \times 10^{-5}$ m s$^{-1}$ from the $y$ intercept of the trend line (Fig. 6).

**Transcuticular Hydrophilic Pores Correspond to the Bases of Trichomes**

Extended TB staining revealed the presence of a limited number of intact trichomes on the fruits, with comparable diameters and distribution patterns to the spots (Fig. 5C). More common, however, were partially broken trichomes, and closer examination of the blue puncta revealed that they frequently had a small stub of broken trichome protruding from them (Fig. 5D), which was visible even after only a 15-min TB treatment (Supplemental Fig. S6, A and B). To examine the morphology of the trichome stubs, we used confocal microscopy to image stained pericarp sections and isolated cuticles (Fig. 7, A and B). Cuticular lipids were stained with Auramine-O (orange in the confocal image), cellulose was stained using Calcofluor White (blue), and overlapping signals appear as pink. Stubs of broken trichomes were observed protruding from the cuticle surface, with a small channel (~5–10 μm diameter) containing polysaccharides, but free of lipophilic material, clearly visible at the base of each (Fig. 7, A and B; Supplemental Movie S1). Analysis of Oil Red O-stained cuticles from the wild tomato relative *Solanum neorickii* revealed the presence of the same channel structure, suggesting that they are common to fruits from across the tomato clade (Fig. 7C).

The abundances of pores observed by TB staining suggested that tomato fruit produce many trichomes but that the density varies between cultivars. We observed that untouched fruit had a trichome-rich surface, from early development through to the fully ripe stage, with the higher-water-loss lines having a higher trichome density (Supplemental Fig. S7). Upon any handling of the fruit, the trichomes

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**Figure 5.** MG fruit stained with TB. A, Blue puncta appeared after 15 min and spread through the underlying tissue after 3 d of staining (inset). B, The permeances of isolated MG cuticles from the seven cultivars correlated with their density of TB staining. $n \geq 5$; means ± se are shown. C, Treatment of fruits with TB for 3 d stained intact trichomes. D, Close examination of the blue spots often revealed the remainder of a broken-off trichome. Insets in C and D show closeups of trichomes. Black bars = 1 mm, and white bars for insets = 200 μm.

**Figure 6.** Permeance of isolated red ripe-stage fruit cuticles from cv M82 mounted in transpiration chambers in relation to the area of the cuticle that was stained after application of TB on the outer face for 2 d. Each point represents a single cuticle sample.
readily broke off, leaving behind the stubs and open pores described above. Importantly, even while still fully intact, the trichome tips were highly permeable and stained readily with TB, indicating a low-resistance route for water flux (Fig. 8).

Fourier-Transform Infrared Spectroscopy Analysis of the Transcuticular Pores

To characterize spatially how the chemical nature of the hydrophobic cuticle differs from that of the pore, we...
used Fourier-transform infrared (FT-IR) spectroscopy to measure IR absorbance spectra at five locations along transects of isolated MG-stage cuticles, with one spectrum collected directly over a TB-stained pore (Fig. 7D). The collection window was slightly too large to collect a signal from only within the pore, so this area also contained part of the trichome base. The pore region showed similar absorbance of wavelengths corresponding to cuticular lipids, compared with the pore-free areas, but had consistently higher absorbance in the polysaccharide regions of the spectrum (Fig. 7D). The ratio of polysaccharide peak area to lipid peak area was 28% higher for the pore region compared with the average of the other four locations. On a peak height basis, the ratio was 49% higher for the pore. This relative increase in polysaccharide-associated absorbance is consistent with the microscopy observations of a lipid-free, polysaccharide-rich pore in the cuticle. The overall absorbance by lipids is not reduced, as their absence in the pore is offset by the additional cuticular material of the trichome base.

**Pore Density during Fruit Ontogeny and Pore Sealing**

We next examined the presence of the transcuticular pores during fruit development by TB staining fruit of cv M82 at the immature green, MG, light red, and red ripe stages (Supplemental Fig. S8). The pore density decreased between the immature and mature stages, as a consequence of fruit expansion. We observed that the pores from immature, expanding fruits had a higher permeance than those of fully expanded fruits, allowing more rapid penetration of TB through the cuticle. This could be because the cuticles of mature fruits are much thicker (Segado et al., 2016), which would mean that the channels are longer, but it might also reflect partial sealing of the pore. We hypothesized that there would be a greater chance of the pores becoming sealed during fruit development if the trichomes had already broken off, since wounding of the fruits is known to trigger suberin production (Dean and Kolattukudy, 1976; Tao et al., 2016). To test this, we thoroughly wiped off the trichomes from one side of green fruit that were midway through the expansion phase and then compared the pore density between the two sides after the fruit had subsequently matured and ripened. TB staining revealed that numerous pores were present on the wiped sides of the fruit; however, their density was lower than on the undisturbed sides (Fig. 9), suggesting that a portion of the pores from the damaged trichomes had indeed sealed during growth and/or ripening.

**Relative Rates of Water Loss through the Cuticle and Stem Scar in Ripe Fruit**

These results suggested that a substantial amount of transpirational water loss from tomato fruit can occur through polar pores in the cuticle, associated with damaged and intact trichomes. However, another potential route of water loss from detached fruit is the stem scar. To test the relative contributions of the cuticle and stem scar to postharvest fruit transpiration, we measured water loss from ripe fruits of each of the seven varieties, with their stem scars unsealed, over the course of 3 weeks of storage. The transpiration rates declined noticeably over this time period, but to differing degrees across the varieties (Supplemental Fig. S9). At no point, however, were the relative water loss rates the same as those observed previously for MG-stage fruits with sealed scars (Fig. 2A). This suggested that exposure of the stem scar and/or ripening of the fruit affected the rate of water loss.

To distinguish between these two possible effects, we also measured the transpiration rates of ripe fruit of two
varieties, 10022 and 10077, with their stem scars sealed. While the transpiration rates of unsealed fruits declined over time, those of the sealed fruits remained fairly constant (Fig. 10A). The difference between the two rates represents the water loss through the stem scars (Fig. 10A, dashed lines). The average water transpiration rate through stem scar tissue was calculated for different lengths of time after harvest, and we observed that initial values of 8.2 mg mm\(^{-2}\) d\(^{-1}\) declined to 1.3 mg mm\(^{-2}\) d\(^{-1}\) during week 3. An assumption was made that these permeance values are consistent across cultivars, allowing the rate of water loss from the fruit stem scars to be calculated based on their area (Fig. 10B and C). We concluded that the stem scar provides a major route for water loss in some cultivars immediately after harvest, but its effect diminishes considerably over week 1 (Fig. 10C; mean for days 1–3 > mean for days 5–7; \(P < 1 \times 10^{-6}\) for each variety). The stem scars do not fully seal, however, even after 3 weeks (Supplemental Fig. S10A). We calculated that the relative contribution of cuticular transpiration to the total transpirational water loss from the fruit increases during storage, from 20% to 67% of the total initially, to greater than 75% during week 2 in all cultivars (Fig. 10D).

By subtracting the expected contribution of the stem scar from the whole-fruit transpiration rate, we could estimate the transpirational flux through the cuticle of each of the unsealed ripe fruits. The average flux for each line in the days immediately after harvest (Fig. 10E, days 1–3) showed the same cross-cultivar trend, within each size group, as that observed previously for the MG-stage fruits (Fig. 2A). However, the absolute values were slightly different: the small fruits had lower cuticular flux at the ripe stage, whereas the medium fruits had either the same or increased values. Over the 3-week time course, the rate of transpirational water flux through the skin decreased gradually in lines 10241 and 10062, increased in line 10177, and showed no statistically significant change (\(P < 0.05\)) in the other lines, although 9999 trended slightly downward and...
10022 and 9959 trended slightly up (Fig. 10E). Thus, the transpirational flux through the fruit cuticle can change during ripening and during storage, but this varies considerably among cultivars.

The postharvest decreases in cuticle transpiration rates seen in some lines are consistent with a degree of postharvest sealing of the transcuticular pores. In support of this, some pores were observed to be filled with lipophilic material in fruit of line 10062 after 3 weeks of storage, while freshly harvested fruit consistently showed open pores (Supplemental Fig. S11, A and B). However, such sealing is not widespread, as TB staining revealed numerous pores in all fruits at the end of the storage period (Supplemental Fig. S10B).

**DISCUSSION**

**Whole-Fruit Transpiration**

In the initial tomato genotype survey, a wide range of fruit transpiration rates was observed. The greatest diversity was seen in *S. lycopersicum*, which is likely a reflection of the considerable intercultivar variation brought about by human selection for numerous fruit traits. The low transpiration rates seen across the *S. pimpinellifolium* accessions suggest that this is the ancestral state, which has been lost during domestication. Indeed, the intermediate *S. lycopersicum cv* *cerasifome* accessions have higher transpiration, suggesting that this property may have arisen as an early consequence of breeding for increased fruit size. It is notable that among the distribution profiles, the peak of *S. lycopersicum* is lower than that of *S. lycopersicum cv* *cerasifome* (Fig. 1), which suggests that water retention became a target for selection later in domestication.

This screen was carried out using MG fruits, as the timing of ripening varied among the many cultivars. Furthermore, fruits are firmer at the MG stage than at full ripe, making them more resistant to damage during harvest, transport, and handling.

Following the initial survey of field-grown plants, in subsequent studies we focused on the lines with the lowest or the highest transpiration rates, and the chosen accessions generally showed the same dichotomy when regrown under controlled growth conditions in the greenhouse. The low-transpiration lines showed the same result as in the initial screen, with the exception of line 10241, which had the lowest transpiration rate of all the *S. lycopersicum* accessions but showed an intermediate phenotype in the confirmation trial. The values from the high-transpiration lines were less reproducible between trials, which we concluded was due to cuticle microfissuring or other unnoticed defects causing spuriously high measurements.

**Differences in Cuticle Permeance Underlie Transpiration Rates**

Since it is possible that transpiration rates could be influenced by internal properties of the fruit, we measured the permeance of isolated cuticles directly and found that the cuticles could indeed account for the transpirational difference between whole fruits of the high- and low-water-loss lines. This is consistent with other studies that have found that the rates of water loss from organs with closed stomata are determined by cuticle permeability (Kirsch et al., 1997; Burghardt and Riederer, 2003; Šantruc, et al., 2004). We report permeance values because this measure is independent of the transpirational driving force and thus is more easily comparable across studies. We note that our calculated values are consistent with those in other studies of tomato fruit cuticles (Schreiber and Riederer, 1996; Leide et al., 2007, 2011).

**Neither Cuticle Abundance nor Composition Explains Permeance**

We investigated whether the differences in cuticle permeabilities could be explained by the amount of total cuticle or specific components per unit area. None of the variables of cuticle penetration depth, cuticle mass, total cutin abundance, or total wax abundance showed a correlation with relative transpiration rates across the seven varieties. There was, however, a correlation for the small-fruited varieties when considered on their own, but it should be noted that the tomato fruit cuticle extends between, and often beneath, the epidermal cells (Fig. 3D; Supplemental Fig. S2), so the total amount of cuticle includes anticlinal pegs and subepidermal deposits, which are unlikely to contribute to the transpiration barrier. Consequently, a more appropriate measure is the thickness of cuticle that overlies the epidermal cells, as this corresponds to the shortest path to the surface. This value showed very little variation across the small-fruited varieties (Fig. 3C) and thus does not account for the differences in their transpiration rates.

There is growing evidence that the composition of the cuticular waxes, and in particular the alkane-to-triterpenoid ratio, is more consequential than the total amount of wax (Vogg et al., 2004; Leide et al., 2011; Parsons et al., 2013). The high wax levels in the two low-fruited varieties re

**Tomato Fruit Cuticle Permeance Is Largely Dictated by Trichome-Associated Transcuticular Polar Pores**

While staining intact fruit with TB, we observed the appearance of blue-stained spots distributed across the
fruit surface, indicating regions of higher hydrophilicity in the cuticle. Closer examination revealed that they occur at the site of trichome attachment to the epidermis. Trichomes have typically not been given much consideration in studies on mature tomato fruit, as they are largely absent after harvest. However, they are abundant if the fruit is undisturbed (Supplemental Fig. S7), and we found no evidence of natural abscission or loss in the absence of handling or other abrasions. Trichomes must maintain a route for water and metabolite supply from the underlying tissue, and thus a continuous hydrophobic cuticle cannot be deposited beneath them. We report the identification of cuticle-free but polysaccharide-containing pores at the base of trichomes that span the over-epidermal cuticle (Fig. 7). Notably, the size of these polar pores in the cuticle (5–10 μm in diameter) is 2 orders of magnitude larger than the 0.5-nm pores that were predicted mathematically for bitter orange (Citrus aurantium) leaf cuticles (Schönherr, 1976). These pores represent hydrophilic channels between the interior and exterior of the fruit, through which water and aqueous solutes could theoretically readily diffuse or flow. Even if an intact trichome remains above the pore, it is unlikely to provide a major barrier, despite having a cuticle coating, due to the high permeability at the tip (Fig. 8; also seen in bean [Vicia faba] leaves [Schreiber, 2005]), which is likely necessary for their secretory functions.

Furthermore, we show that the abundance and conductance of these trichome-associated pores account for most of the variation in cuticular permeances between the seven cultivars (Fig. 5B; $R^2 = 0.96$) and between individual cuticle samples (Fig. 6; $R^2 = 0.84$). The pores provide a low-resistance route for water movement through the cuticle, allowing for considerably more rapid cuticular transpiration. We propose that the remaining variation in permeance that is not explained by the pores is likely an effect of cuticle amount or composition.

**Trichome Pore Tradeoffs**

Our findings suggest that the presence of trichomes accelerates postharvest desiccation, but their retention throughout evolution and domestication indicates that they have a beneficial role. The primary function typically ascribed to tomato trichomes is as an anti-herbivory mechanism, reducing insect colonization and damage on the stems and leaves (Simmons and Gurr, 2005; Tian et al., 2012). However, it is not evident that this role extends to the fruit of cultivated tomato, where the trichomes are comparatively small and sparse at maturity. Indeed, trichomes may increase the susceptibility of fruit to microbial infection by serving as entry sites for pathogenic bacteria (Getz, 1983; Barak et al., 2011).

We propose an alternative hypothesis for the retention of fruit trichomes throughout tomato evolution and domestication: that the increase in cuticular permeability as a consequence of their associated pores is beneficial for fruit physiology and promotes consumption by frugivores. First, these large permeable pores would allow volatiles to be released from the fruit more rapidly, enhancing the attraction of consumers/ dispersal vectors. Second, a higher rate of transpiration from the growing fruit would result in greater influx of xylem sap, increasing the acquisition of mineral nutrients, such as calcium (Hocking et al., 2016). This could be critical for larger tomato fruit, which develop the physiological disorder blossom end rot in the absence of sufficient calcium (Taylor and Locascio, 2004; Hocking et al., 2016). Indeed, line 10241, which produced medium-sized fruit with low transpiration, was observed to be highly susceptible to blossom end rot.

Our observation that the cuticular pores can become sealed after trichome detachment suggests a possible evolutionary adaptation that minimizes their functional tradeoffs. Since the proposed beneficial effects would be most important before the fruit is mature, and deleterious effects would be manifested after maturation, perhaps the ideal fruit would have numerous trichomes but also a high rate of pore sealing once the fruit ripens. The pores appear to decrease in conductance once the fruit is fully expanded, suggesting that sealing occurs during, or on completion of, fruit expansion. The differences in cuticular transpiration flux between MG and freshly harvested ripe tomato fruit could suggest that pore sealing also occurs during ripening; however, ripening-associated changes in cuticle composition (Leide et al., 2007; Romero and Rose, 2019) may also make a contribution. In addition, we found evidence of postharvest pore sealing, although its occurrence appeared to be cultivar dependent. This indicates that a mechanism exists for sealing pores in ripe fruits, which presents an intriguing target for breeding or genetic engineering efforts.

**The Cuticle, Not the Stem Scar, Is a Main Contributor to Postharvest Water Loss**

Although the cuticle covers most of the surface of a tomato fruit, a small patch of tissue is exposed when the stem is removed and this could potentially be a major route of water loss. We found that the stem scars were a contributor to the overall transpiration rate of the fruit but that their contribution was less than that of the cuticle, except for the first few days after harvest in some of the cultivars (Fig. 10D). The rapid decline in the stem scar transpiration rate indicates that they undergo healing to seal the damaged surface. This was observed by Leide et al. (2012), who described extensive suberization of the stem scar and underlying tissue after harvest. The authors also observed a substantial decline in stem scar transpiration in parallel with suberin deposition. The cuticle can therefore be considered as the major boundary across which transpiration occurs during storage of tomato fruit.
CONCLUSION

In this study, we report variability in fruit transpiration rates across tomato cultivars and show that the extremes of this variation reflect differences in the density of trichome-associated transcuticular pores. Their effect on water flux considerably outweighs any contributions from differences in cuticular chemical composition. Since water loss, along with pathogen infection, are two of the major causes of postharvest spoilage, increasing the ability of the mature tomato fruit to seal these pores could serve as an effective means to improve food quality and decrease wastage. Whether a similar mechanism occurs in other fruits has yet to be determined.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The tomato (Solanum lycopersicum) diversity panel was grown in the ground in a greenhouse in Rehovot, Israel, over the winter of 2014-2015. The lines of interest were then regrown in 3-gallon pots of LM-111 soil mix (Lambert) in a greenhouse in Ithaca, New York, with a 16-h daylight. The accession identifiers used here refer to the following cultivars: 9959, 'Cherry Roma' (EA006889); 9999, 'FLD 56' (EA07181); 10022, 'DZ 76' (EA0720); 10062, 'Ida' (EA0362); 10077, 'LA1482' (EA07003); 10177, 'LA0395' (EA0058); and 10241, 'Vito' (EA01853). Most analyses were performed using MG-stage fruits, defined as those that have reached the size of ripe fruit but have yet to show any color change. Immature green fruit are defined here as green fruit with a diameter approximately half that of a mature fruit. Light red fruit showed an orange color over at least 90% of the fruit surface, while ripe red fruits were entirely a dark red color.

Fruit Water Loss Rate and Toluidine Blue Staining

For the initial panel screen, six MG-stage fruit were harvested from each of 398 different tomato accessions (Supplemental Table S1). The fruit were rinsed with water and dried, and their stem scars, along with any noticeable cracks or blemishes, were sealed with vacuum grease (Dow Corning). They were then placed in trays in an open room, with the six fruit of each line divided between two locations in the room. Over the course of the experiment, the ambient temperature varied from 10°C to 24°C and the relative humidity varied from 37% to 64%. Fruit weight was measured 1 d after harvest and again 3 d later. Fruit that showed obvious signs of damage were discarded. Water loss rate was determined for each fruit as the total change in mass divided by 3 d and by the surface area of the fruit (calculated from the average of three diameter measurements).

For all other such experiments, fruit were prepared and analyzed as above but were stored in a controlled-atmosphere chamber at 22°C and 30% relative humidity. In the ripe-fruit trial, only those fruit specifically indicated had their stem scars sealed with vacuum grease. Weight measurements were taken 1 and 4 d after harvest as well as at the other indicated time points for the ripe-fruit trial. After the final weight measurements, fruit were immersed in 0.05% (w/v) solution of TB for 15 min, then rinsed in deionized water and imaged with a Stermi 508 stereo microscope (Zeiss), and the number of stained spots was counted (Supplemental Fig. S5). Cuticle sample showing fissures or holes were discarded. At least five cuticle samples were imaged for each line. For quantification by TB-stained area (Fig. 6), the cuticles were stained for 2 d, then rinsed and photographed as above. The total area stained blue was determined from the images using ImageJ software (imagej.nih.gov). The threshold function was used to select only pixels with a hue in the blue or green range (background cuticle color is orange), then these pixels were counted with the Analyze Particles function.

Wax Extraction

MG-stage fruits were harvested and rinsed in deionized water. A metal probe was inserted into each fruit through the stem scar, and holding the probe, the fruit was swirled in a beaker of ~100 mL of chloroform, containing 100 μg of tetradecanoate as a standard, for 90 s. Each chloroform aliquot was used to extract wax from two fruit and represented one biological replicate. The chloroform extracts were concentrated by air drying, then filtered through filter paper (VWR) preirradiated with chloroform. There were five biological replicates per line.

Cutin Isolation

A disc, 7.4 mm in diameter, was cut with a cork borer from the peel of each of the fruits used for wax extraction and the cuticle was isolated as described above. The dry isolated cuticle discs were sequentially rinsed in chloroform, 2:1 chloroform:methanol, 1:2 chloroform:methanol, and pure methanol, shaking for 20 min in each. The cutin was then depolymerized following the base catalysis method (Li-Beissner et al., 2013). Each cuticle disc was added to a vial containing 1 mL of reaction medium (12:3:5 methanol:methyl acetate:25% (w/v) sodium methoxide) and 50 μg each of pentadecalcotane and heptadecanoate, as internal standards. The samples were incubated at 60°C overnight, cooled to room temperature, and 2 mL of dichloromethane, 0.25 mL of glacial acetic acid, and 0.5 mL of 0.9% (w/v) NaCl in 100 mM Tris (pH 8) were added to each. The samples were vortexed and then centrifuged for 2 min at 1,500g. The lower phase was transferred to a clean vial containing 1 mL of 0.9% (w/v) NaCl in 100 mM Tris (pH 8). This was vortexed and centrifuged (2 min at 1,500g), and the lower phase was transferred to a clean vial. Approximately 0.5 g of anhydrous sodium sulfate was added to bind any water present, and the solution was then filtered through a paper filter (Whatman no. 1) into a clean vial. A total of three biological replicates were analyzed per line.

Wax/Cutin Analysis

An aliquot of each sample was dried by heating at 40°C under a stream of N2. The dried aliquots were derivatized by adding 50 μL of each pyridine (EMD}

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Millipore) and N,O-bis(trimethylsilyl)trifluoroacetamide (Sigma) and heating for 30 min at 70°C for the wax samples or for 10 min at 90°C for the cutin samples. These were dried under N₂ as before, resuspended in 100 μL of chloroform, and then analyzed by GC using a GC 6850 (Agilent), as described by Martin et al. (2017). Compound abundances were determined by integration of the chromatograph peaks using the ChemStation software (Agilent) and normalization to the internal standard and to the surface areas of the samples. The identity of each compound was determined based on a comparison of retention times with standards and also by GC-mass spectrometry analysis, using a GC 6890 (Agilent) coupled to a JOEL GC MATE II mass spectrometer.

Cuticle Thickness and Weight
Cuticle thickness was measured from images of Oil Red O-stained pericarp sections (Fig. 3D) using ImageJ software. Over-epidermal cell thickness was measured over the midpoint of epidermal cells that appeared full sized (i.e. sectioned through their middle). Penetration depth was measured from the surface to the plane representing the deepest penetration of cuticular material. Both measures were taken in three or four places per image, with three images taken along the cuticle from each of six fruits per line. The mean of the values was taken for each cuticle to give six biological replicates. The variation across the six replicates was used to calculate the s.e. for that line. The weights of squares of dry isolated cuticles were measured and normalized to their area (n ≥ 8).

Staining and Microscopy
Blocks of outer pericarp from MG fruit were fixed, embedded, and cryo-sectioned as previously described (Buda et al., 2009). The sections were bound to Histobond slides (VWR) by heating at 100°C for 3 min. The embedding medium was rinsed off with deionized water, and the sections were stained with Oil Red O (Alfa Aesar) in 60% (v/v) isopropanol for 30 min. Destaining was performed by dipping slides in a series of five isopropanol solutions decreasing from 55% to 8% (v/v) isopropanol. Sections were mounted in deionized water and imaged using an Axio Imager A1 microscope (Zeiss).

For confocal microscopy, MG pericarp sections 30 μm thick, along with isolated cuticles, were stained sequentially with Calcofluor White M2R solution (Sigma) for 2 min, then 0.01% (w/v) Auramine-O (Sigma) in 0.05 M Tris, pH 7.2, for 15 min, then rinsed with deionized water. Samples were mounted on a slide in water imaged with an LSM 710 confocal microscope (Zeiss), using a 40× water-immersion objective. The Auramine-O fluorescence was induced with an excitation wavelength of 458 nm and emission collected from 481 to 639 nm. The Calcofluor White fluorescence was induced with an excitation wavelength of 405 nm and emission collected from 481 to 443 nm. Three-dimensional reconstructions were generated using Zen software (Zeiss) with 50 to 50 images in a Z-stack. Supplemental Movie S1 was created using Vision4D software (Artvis); the opacity of the structures was increased for illustrative purposes. All the confocal images have false coloration.

FT-IR Analysis
IR spectroscopy was performed using a Bruker Hyperion microscope-coupled FT-IR spectrometer in transmission mode at the Cornell Center for Materials Research (www.ccmr.cornell.edu). Isolated cuticles were stained on the outer surface with TB solution for 15 min, rinsed, and then air dried. IR absorption measurements were taken at five points along a transect, with the center point being a transcuticular pore (identified based on the TB staining). The area to be analyzed for each point was demarcated using IR-opaque plates. Spectra were baseline normalized. Peaks were assigned based on previous reports (Vilena et al., 2000; Heredia-Guerrero et al., 2014; Fasoli et al., 2016). Peak area was measured over 950 to 1,200 cm⁻¹ for polysaccharides and over 1,700 to 1,745 cm⁻¹ plus 2,780 to 3,000 cm⁻¹ for the lipids. Peak height was measured as the average height of the two tallest peaks for each compound class (2,928 and 1,752 cm⁻¹ for lipids and 1,167 and 1,036 cm⁻¹ for polysaccharides).

Statistical Analyses
Quantitative measures are presented as means ± SE. In Figures 2 and 10, the means of the varieties were compared using ANOVA with Tukey's posthoc tests. Correlations between permeance and spot density or area were tested with linear regression. These analyses were performed using the statistical package R (r-project.org). One-tailed Student's t tests were carried out in Microsoft Excel to compare the days 1 to 3 and days 5 to 7 means shown in Figure 10C.

Supplemental Data
The following supplemental materials are available.

Supplemental Figure S1. Schematic of the transpiration chamber setup for measuring cuticular permeance.

Supplemental Figure S2. Light microscopy images of the cuticle of MG-stage fruit from the seven targeted varieties, stained in red with Oil Red O.

Supplemental Figure S3. Relative abundances of cutin monomers from each line.

Supplemental Figure S4. Relative abundances of the cuticular wax components detected by GC.

Supplemental Figure S5. Isolated cuticle mounted in a transpiration chamber and stained with TB.

Supplemental Figure S6. Broken trichome stubs on the surface of tomato fruit stained with TB for 15 min.

Supplemental Figure S7. Undisturbed MG and ripe fruit of the seven tomato varieties.

Supplemental Figure S8. Cultivar M82 fruit at four developmental stages stained with TB for 20 min, then for 15 h.

Supplemental Figure S9. Rate of water loss from whole ripe fruits of the seven varieties, with their stem scars unsealed, during the first 2 d and week 3 after harvest.

Supplemental Figure S10. TB staining of ripe fruits following postharvest storage.

Supplemental Figure S11. Pores show evidence of sealing in ripe fruits following postharvest storage.

Supplemental Table S1. Tomato lines tested in the initial fruit transpiration assay.

Supplemental Movie S1. 3D visualization of cv M82 fruit upper pericarp with a trichome stub and associated cuticular pore.

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