A novel class of *sticky peel* and *light green* mutations causes cuticle deficiency in leaves and fruits of tomato (*Solanum lycopersicum*)

Journal:	Planta
Manuscript ID:	Planta-2012-04-0276.R3
Manuscript Type:	Original Article
Date Submitted by the Author:	08-Jul-2012
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Keywords:	Cutin, Epidermis, Wax

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10 4 A novel class of *sticky peel* and *light green* mutations
11 5 causes cuticle deficiency in leaves and fruits of tomato
12 6 (*Solanum lycopersicum*)
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Abstract

The plant cuticle consists of aliphatic wax and cutin, and covers all the aerial tissues, conferring resistance to both biotic and abiotic stresses. In this study, we performed phenotypic characterizations of tomato mutants having both *sticky peel* (*pe*) and *light green* (*lg*) mutations. Our genetic analysis showed that these two mutations are tightly linked and behave like a monogenic recessive mutation. The double mutant (*pe lg*) produced glossy soft fruits with light green leaves, most likely due to defects in cuticle formation. Cytological analysis revealed that the thickness of the fruit cuticle layer was dramatically reduced in the *pe lg* mutant. The epidermal cells of the leaves were also deformed in the *pe lg* mutant, suggesting that leaf cuticle formation was also disrupted in the mutant. Consistent with this, transmission electron microscopic analysis showed that the electron density of the cuticle layer of the adaxial surface of the leaf was reduced in the *pe lg* mutant compared to WT, suggesting that there are changes in cuticle structure and/or composition in the *pe lg* mutant. Both physiological analysis to measure the rate of transpiration, and staining of the fruits and leaves with toluidine blue, revealed that water permeability was enhanced in the *pe lg* mutant, consistent with the reduced thickness of its cuticle layer. Taken together the preliminary analyses of the cuticle components, the *PE LG* is most likely involved in proper cuticle formation.

Keywords: Cutin, Epidermis, Wax

Abbreviations

cv, cultivar
DMF, N,N-dimethylformamide
lg, *light green*
pe, *sticky peel*
TB, toluidine blue
SEM, scanning electron microscope
TEM, transmission electron microscopy analysis
TGRC, Tomato Genetic Resource Center
WT, wild type

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3 **1 Introduction**
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3 The epidermal tissues of plants play many important roles in protecting them from mechanical
4 damage, drought, pests, and diseases caused by fungi and bacteria. (Riederer 2006). This protection is
5 afforded by the cuticle, which consists of lipidic cuticular wax and polymeric cutin. Cuticular wax
6 consists of epicuticular wax, which resides on the outer surface of the epidermis, and intracuticular wax,
7 which is interspersed in the cutin (Jetter et al. 2006). Epicuticular wax provides resistance to biological
8 stressors such as insects and fungi (Samuels et al. 2008), and intracuticular wax is thought to be
9 involved in cuticular transpiration (Vogg et al. 2004; Leide et al. 2007). Biochemical analyses of
10 cuticular wax show the presence of various alcohols, esters, aldehydes, alkanes, and ketones with long
11 (>C20) straight aliphatic chains (Samuels et al. 2008).

12 Cutin, however, is a structural component of the epidermis and physically protects plant
13 tissues against the entry of disease agents, while maintaining adequate cuticular transpiration and gas
14 exchange (Pollard et al. 2008). Cutin has been shown to consist of fatty acids, dicarboxylic acids, and
15 alcohols with C16 or C18 carbon chains linked to glycerol and acylglycerols (Graça et al. 2002; Li and
16 Beisson 2009). Although the biological functions of cutin have been widely investigated, its
17 biosynthesis is not still completely understood.

18 In *Arabidopsis*, forward and reverse genetic approaches have enabled a partial understanding
19 of the biosynthetic pathways of wax and cutin, as well as their physical functions (Yephremov and
20 Schreiber 2005). Mutants with defective wax biosynthesis typically show a decrease in the amount of
21 wax and/or an alteration in wax composition (Samuels et al. 2008). Furthermore, a phenotype with
22 fused postgenital organs is typically observed in these mutants (Lolle et al. 1998). Mutants showing
23 defective cutin biosynthesis generally have decreased levels of cutin monomers (Xiao et al. 2004; Li et
24 al. 2007), and altered levels of the various wax components (Schnurr et al. 2004), suggesting a
25 biochemical interaction between cutin and wax synthesis. Several lines of evidence have suggested
26 that a reduction in wax and cutin content is likely to be associated with increased cuticle permeability
27 and susceptibility to pathogens (Xiao et al. 2004; Li et al. 2007). In contrast, disease resistance is
28 enhanced in the *long-chain acyl-CoA synthetase2 (lacs2)* and *sitiens* mutants which exhibit defective
29 cutin biosynthesis (Bessire et al. 2007; Curvers et al. 2010), suggesting that the physical ability of the
30 cuticle to resist disease is ambiguous and that different states of permeability may exist in plants.

In tomato fruit, cutin and wax play important roles in water permeability and physical protection, similar to that reported in *Arabidopsis* (Bargel et al. 2005). For example, the fruit of the *delayed fruit deterioration* (*dfd*) mutant is highly resistant to water loss, resulting in an extremely long shelf-life, although the ripening process appears to be normal (Saladié et al. 2007). Fruit of the *dfd* mutant is also resistant to fungal invasion. The cuticle layer in the *dfd* mutant is probably responsible for these phenotypes because physical damage (e.g. scratching) of its cuticle layer results in increased susceptibility to dehydration and fungal attack. The total wax content in the *dfd* mutant fruit is about 1.3-fold higher than that in the corresponding wild-type (WT) cultivar Ailsa-Craig and, particularly, the cutin content in the *dfd* mutant fruit is almost double that of WT. Unlike the *dfd* mutant, several tomato mutants show an increase in water permeability of the fruit. For example, the fruits of the *Lycopersicon esculentum eceriferum6* (*lecer6*) mutant lack the C31 alkane component in the wax and exhibit increased water permeability and wrinkling of the fruit surface structure during ripening (Vogg et al. 2004; Leide et al. 2007). These observations suggest that the levels of waxes and/or cutin accumulation in fruit are correlated with the levels of water retention and shelf-life. However, the *Cuticular water permeability1^{hir}* (*Cwp1^{hir}*) gene derived from the wild species *Solanum habrochaites* is associated with increased water permeability of the fruit, even though the cuticular constituents, including waxes and cutin monomers, do not significantly differ from those of WT (Hovav et al. 2007). Similar to the *Cwp1^{hir}* phenotype, the fruit cutin content does not seem to be correlated with the permeability of the cuticle to water in the *cutin deficient* (*cd*) 2 mutant (Isaacson et al. 2009). These conflicting results suggest the involvement of distinct mechanisms in the biosynthesis and function of the tomato fruit cuticle. The physical and molecular relationships between cutin and transpiration therefore require clarification.

In this study we report the characterization of a novel tomato cuticle mutant called *sticky peel* (*pe*), which was first reported in Texas in 1938 (Young 1941). The fruit of the '*sticky peel*' tomato resembles that of a cherry in texture; it is much softer than the fruit of WT parental cultivars, and the skin is extremely thin and elastic. The *pe* mutation produces a more elastic fruit surface, which is highly resistant to cracking (Ho and Hewitt 1986), and this trait appears to be related to the cuticular development process. The *light green* (*lg*) mutation is responsible for giving a bright pale green color to the leaves (Kerr 1982). Although previous genetic studies have suggested that both *pe* and *lg* are independent, monogenic recessive mutations (Butler 1952), our genetic analysis indicates that the *pe*

1 and *lg* mutations are tightly linked. Our cytological and physiological analyses show that the *pe lg*
2 mutant belongs to a novel class of cuticular-related mutants which cause defects in both fruit and leaf
3 cuticle layers, resulting in thinner cuticles and increased rates of transpiration.
4

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Materials and methods

Plant materials

We used *Solanum lycopersicum* cv. KGM942, into which both the *pe* and the *lg* mutations have been introduced during breeding. Both mutations were introduced from an accession LA0759 that was obtained from the Tomato Genetic Resource Center (TGRC) (<http://tgrc.ucdavis.edu/>). The F₂ populations were created by crossing KGM942 (*pe/pe*, *lg/lg*) with the corresponding wild type Rejina (*PE/PE*, *LG/LG*, Sakata Seeds, Yokohama, Japan). Other wild-type breeding lines K091, RC17 and Ec-1 were also used as controls to compare cuticle phenotypes. Genotypes in all cultivars are *PE/PE*, *LG/LG*. Besides, K091 carries the *y* mutation known to influence epidermis color due to a lack of naringenin chalcone (Adato et al. 2009; Ballester et al. 2010).

Histological observations

Epidermis sections of ripe red fruits of the *pe lg* mutant were prepared for histological analysis by slicing the fruit horizontally with a sharp scalpel. The sections were observed under a scanning electron microscope (SEM, x 500, TM-1000, Hitachi High-technologies) without fixation and coating, according to the manufacturer's protocols. The cuticle layer was observed after staining with Sudan IV as described by Buda et al. (2009). For leaf cytological analysis, the fourth or fifth leaves were removed and cut into small pieces (1 cm x 1 cm) and the transverse leaf sections were observed under SEM (x 1000) to examine the ultrastructure of the leaf surface. In this experiment, the wild-type cultivar RC17 was used as a control.

Transmission electron microscopy analysis

Tenth to thirteenth leaves were pre-fixed in solution A (2% paraformaldehyde, 2% glutaraldehyde in 50 mM sodium phosphate, pH 7.0) on ice. The leaves were washed with 50 mM sodium phosphate (pH 7.0) for 1 h. Post-fixation was conducted in 2% osmium tetroxide in 50 mM sodium phosphate (pH 7.0) for 3 h on ice. Dehydration was performed via a series of graded ethanol solutions (30% x1, 50% x1, 70% x1, 80% x1, 90% x1, 95% x1, 100% x3) for 30 min each, followed by substitution with propylene oxide for 30 min and a mixture of propylene oxide and epoxy resin for 1 h. The leaves were embedded into a gelatin capsule and incubated with Quetol651 Epoxy resin at 60°C for 2 days. 60–80 nm ultrathin

sections were made using a Leica-UCT ultramicrotome. The ultrathin sections were mounted on copper grids (200 mesh) and stained with 2% uranyl acetate in dionized water for 5 min, followed by incubation with lead staining solution for 5 min. Microscopic observations were carried out using a JEM1200EX (Jeol) at 80 kV.

Chlorophyll measurements

Chlorophyll contents were measured as described by Porra et al. (1989). Five pieces from the fifth leaves of five different plants grown under 16-h light/8-h dark conditions in a greenhouse were collected. The leaves were immersed in 3 ml of N,N-dimethylformamide (DMF) at 4°C overnight. We calculated the concentration of total chlorophyll in the fresh leaf tissue, using the following equations: total micrograms of chlorophyll *a* per gram = 12.00(A664) - 3.11(A647); total micrograms of chlorophyll *b* per gram = 20.78(A647) - 4.88(A664).

Toluidine-blue (TB) tests

To examine water permeability of the plant surface, TB tests were carried out as described by Tanaka et al. (2004). The TB test enables detection of surface defects in a leaf. Second or third leaves and mature green stage fruits were prepared from plants grown in a greenhouse. The fruits and leaves of cultivar RC17 were used as WT.

Transpiration measurements

Transpiration rates were measured to evaluate the water permeability of the leaf epidermis. Five pieces of the tenth to fifteenth leaves from the top of 80-day-old plants were used for this transpiration analysis. The intact leaves were treated with 500 mg/l abscisic acid (ABA) for 1 h before sampling to promote stomatal closure (Freitas et al. 2011), and they were then incubated under constant dark conditions at 25°C as described by Wilkinson et al. (1998). Leaves treated with ABA were checked under a stereomicroscope (SZX7, Olympus) to confirm stomatal closure. The transpiration measurements were conducted over 4 h, and the fresh weights were determined hourly. After the measurements, all leaves were oven dried, and dry matter contents (dry weight divided by fresh weight) determined for each sample. The rate of fresh weight loss was compared between the *pe lg* mutant and WT. The leaves of cultivars K091, RC-17, and Ec-1 were used as controls. To compare the rate of

water loss in fruits, five ripe red fruits were prepared and incubated at 40°C for 6 days. The fruit weights were measured at 1, 2, and 6 days after incubation. Eventually all samples were completely oven dried, and dry matter contents determined for each sample.

Measurement of density in stomata and trichome

To calculate the density of stomata and trichomes, fifth leaves were observed under the SEM. Images of 0.23 mm² leaf area were taken from 5 distinct leaves from both adaxial and abaxial surfaces of each cultivar.

Chlorophyll leaching assays

The barrier function of the epidermis is often disrupted in mutants with defective cuticular development. This disfunction can be easily evaluated using a chlorophyll leaching assay (Lolle et al. 1998) and we conducted chlorophyll leaching assays following Lolle et al. (1997). We collected five pieces of fourth or fifth leaves (approximately 0.2 g each) from plants grown under 16-h light/8-h dark conditions in a greenhouse. The intact leaf samples were soaked in 30 ml of 80% ethanol at room temperature, agitating them in a dark room for 8 h at 37°C (200 rpm). During the immersion, 400 µl of supernatant were taken after 1, 2, 3, 4, 5, 6, and 8 h. The absorption spectrum of each supernatant at 664 and 647 nm was examined to determine the chlorophyll content following Hiscox and Israelstam (1979). We calculated the concentration of total chlorophyll in the fresh leaf tissue, using the following equation: total micromoles of chlorophyll per gram = 7.93(A₆₆₄) + 19.53(A₆₄₇).

Measurement of naringenin and naringenin chalcone

Naringenin and naringenin chalcone were measured as described by Kraus and Galensa (1992), using a L7420 UV-VIS detector (Hitachi) and SUMIPAX ODS-D-05-4615 (SCAS)

Metabolite fingerprinting of cuticular wax and cutin monomer fractions

Metabolite fingerprinting of cuticular wax and cutin monomer fractions was performed as described in Adato et al. (2009) with modifications. Tomato fruits of the *pe lg* mutant and the three breeding lines (Ec-1, RC17 and K091) were boiled and peeled because the peels of the mutant were too fragile for typical enzymatic extraction. To obtain the chloroform-soluble cuticular wax fractions, the manually

1 dissected peel (approx. 20 mg) per sample was put into a 5-ml vial and extracted three times in 2 ml of
2 chloroform (HPLC grade; Wako Pure Chemical Industries) for 1 h at room temperature. Solutions were
3 combined and 0.9 µg of *n*-heptacosane was added to each solution as an internal standard at a final
4 concentration of 15 ng/µl per injection of gas chromatography–electron ionization–time-of-flight–mass
5 spectrometry (GC-EI-TOF-MS), and then the extracts were evaporated to dryness under a stream of
6 nitrogen gas and the dried residue was dissolved in 1 ml of chloroform. Two-hundred-µl aliquot (ca. 4
7 mg of the peel sample) was prepared for GC-EI-TOF-MS analysis by derivatization using *N*-methyl-*N*-
8 (trimethylsilyl) trifluoroacetamide (MSTFA).

9 After the wax extraction, we added 1.8 µg of *n*-heptacosane as an internal standard (a final
10 concentration, 15 ng/µl per GC-EI-TOF-MS injection) to the remaining peels, respectively. Then the
11 cutin samples were depolymerized by adding 1 ml of 10% Boron trifluoride-methanol solution (Fluka)
12 for 1 h at 70°C. After cooling the vials to room temperature, we added 1 ml of Milli Q water. The cutin
13 monomer faction was extracted three times with diethylether (Wako Pure Chemical Industries). The
14 solvent of the combined extracts was removed under a stream of nitrogen gas and the dried residue was
15 dissolved in 1 ml of chloroform. One-hundred-µl aliquot (ca. 2 mg of the peel sample) was prepared
16 for GC-EI-TOF-MS analysis by derivatization using MSTFA.

17 For the fraction solutions containing wax and cutin monomers, chloroform was evaporated from the
18 samples under a stream of nitrogen gas. Subsequently, the wax or cutin fractions were
19 trimethylsilylated for 1 h by 30 µl of MSTFA in pyridine (30 µl) at 37°C with shaking. We conducted
20 metabolite fingerprinting of wax and cutin factions by using GC-MS as described in Kusano et al.
21 (2007b). The chromatograms were pre-processed using the HDA method (Jonsson et al. 2006) and the
22 normalized responses of peak areas were corrected using a constant amount of the internal standard
23 compound (*n*-heptacosane). Structural estimation of cutin and wax monomers was performed by
24 comparing mass fragmentation patterns and the corresponding retention indices of monomers with
25 those in the NIST/EPA/NIH mass spectral library (NIST08), Golm Metabolome Database or our
26 custom library (Stein et al.1999; Kopka et al. 2005) as described in Kusano et al.(2007a).

27
28 Statistical analysis

29 All comparisons among means (ANOVA and Tukey's test; *P*= 0.05) were used to determine
30 differences between the cultivars. Data are presented as means and the standard deviation (SD) with a

level of significance of 5% ($P=0.05$).

2

Results

4

Genetic linkage between the *lg* and *pe* phenotypes

In a previous study it was reported that the *pe* phenotype could be segregated from the *lg* phenotype (Kerr 1982). In order to confirm whether the *pe* and *lg* mutations could in fact be segregated, 334 F_2 populations from a cross between KGM942 (*pe lg*) and Rejina (corresponding WT) were sown in a greenhouse and their phenotypic segregation was examined. We found 252 plants to be completely normal, while 82 showed visible mutant phenotypes derived from both the *pe* and *lg* mutations, indicating that *pe* and *lg* are in fact tightly linked. This co-segregation ratio was estimated as $0.754:0.246 = 3.07:1$, suggesting that the *pe lg* double mutations behave as a monogenic recessive mutation (Table 1). In addition, our preliminary genetic observations showed that, out of more than 1,000 F_2 populations, none of the plants showed either the *pe* phenotype without the *lg* phenotype, or the *lg* phenotype without the *pe* phenotype (data not shown). Our results did not support the claim that both the *pe* and the *lg* mutations are independent monogenic mutations (Kerr 1982). We therefore decided to investigate the double *pe* and *lg* mutant further. We first determined the overall plant phenotype of the *pe lg* double mutant. As far as we are aware, the genetic background of the *pe lg* mutant is unknown and not referred to in any of the literature. We therefore used three wild-type (WT) breeding lines, K091, RC17 and Ec-1 because these cultivars show similar morphological features overall. It is most likely that the *pe lg* mutations do not directly influence fresh weight and the size of leaves and fruits (Supplemental Fig. S1), but other differences in genetic background do seem to exert effects on these traits. In addition, like WT cultivars, the *pe lg* mutant does not cause an organ fusion phenotype and sterility (data not shown).

25

Abnormal epidermis formed in the fruit and leaf of the *pe lg* mutant

The fruit surface of the *pe lg* mutant was shiny and glossy, with increased elasticity compared with WT (Fig. 1a and 1b). In addition, the fruit peel of the *pe lg* lacked the yellow-colored pigment of WT (Fig. 1c and 1d). The lack of yellow pigment is known to be caused by decreased accumulation of the yellow flavonoid pigment, naringenin chalcone, which also influences the physical function of the

1 cuticle layer (Adato et al. 2009; Ballester et al. 2010). We then analysed whether naringenin
2 accumulation was modified in the *pe lg* mutant. Three WT cultivars were used for comparison. K091
3 produces yellow-deficient peel due to the presence of a *y* mutation that reduces accumulation of both
4 naringenin and naringenin chalcone (Adato et al. 2009; Ballester et al. 2010). The experiment showed
5 that the naringenin concentration in the *pe lg* mutant was much lower than in WT, but was not
6 significantly different from K091 carrying the *y* mutation, indicating that the *pe lg* mutations affect
7 flavonoid accumulation (Supplemental Fig. S2).

8 In order to discover whether the difference in fruit surface phenotype was due to differences in the
9 structure of the epidermal cell layers, the surface of the fruit peel of WT plant and the *pe lg* mutant
10 were observed using SEM (Fig. 1e, 1f). A smooth epidermis was formed in WT plants (Fig. 1e). In
11 contrast, the partitions between each epidermal cell (Fig. 1f) were visible in the *pe lg* fruit. When
12 horizontal sections of the fruit epidermis were observed, the fruit epidermis of WT plants consisted of
13 epidermal cells embedded in the cuticle layer (Fig. 1g, arrowhead). In contrast, the epidermis of the *pe*
14 *lg* fruit consisted of epidermal cells alone (Fig. 1h, arrowhead) and the cuticle layer appeared to be
15 much thinner than that in WT. To examine the thickness of the cuticle layer, we attempted to stain the
16 lipidic cuticle layer using SudanIV (Buda et al. 2009). In WT fruit, a thick cuticle layer was visible on
17 the epidermal cells (Fig. 1i, see arrowhead). However, a much thinner cuticle layer was observed on the
18 epidermal cells in the *pe lg* mutant (Fig. 1j, see arrowhead), most likely due to reduced cuticular
19 components.

20 To examine the effect of the *lg* mutation on leaf phenotypes, the leaf surfaces of the *pe lg* mutant and
21 WT cultivar (RC17) were observed (Fig. 2). The leaf color of the *pe lg* mutant was a paler light green
22 compared with that of WT (Fig. 2a and 2b), suggesting that the quantity of chlorophyll was reduced in
23 the *pe lg* mutant. To examine this possibility, we measured the chlorophyll contents of the *pe lg* mutant
24 and WT plant. However, we found no significant differences in chlorophyll *a* content between the
25 mutant and the two WT cultivars (K091, RC17), whereas chlorophyll *b* content was slightly higher in
26 the *pe lg* mutant compared with that in the three WT cultivars (Supplemental Fig. S3). This suggested
27 that the paler leaf color in the *pe lg* mutant was not caused by decreased levels of chlorophyll content.
28 It is known that some cuticular defects alter the reflection of light at the leaf and the stem surface
29 (Aarts et al. 1995; Tarumoto 2004). More research is needed to clarify whether the pale leaf color of the
30 *pe lg* mutant results from a difference in light reflectance due to the thinner cuticle layer, changes in

epidermis structure, or increased chlorophyll *b* content.

We next observed the abaxial surface of leaves using SEM to examine whether there were any structural differences between the mutant and WT. The guard cells of WT plants were half moon-shaped (Fig. 2c), whereas those of the *pe lg* mutant were crescent-shaped (Fig. 2d). In addition, the guard cells and the epidermal cells appeared twisted in the mutant. Some *Arabidopsis* mutants with defective cuticles show similar structural differences in their epidermal cells (Chen et al. 2003; Schnurr et al. 2004; Xiao et al. 2004; Kurdyukov et al. 2006; Li et al. 2007) and in their guard cells (Li et al. 2007). These results implied that the leaf cuticular structure might be altered in the *pe lg* mutant. To examine this further, leaf cross-sections were prepared and observed using light and transmission electron microscopy (TEM) (Fig. 3). Under light microscopy, no differences were observed between the cellular structure of three WT cultivars (Fig. 3a, 3b, 3c) and the *pe lg* mutant (Fig. 3d). From the adaxial surface of the leaf, the adaxial epidermis, palisade parenchyma, spongy mesophyll (parenchyma), and abaxial epidermis were similarly observed in both WT cultivars and the *pe lg* mutant. When the sections were observed using TEM, the adaxial surface of the leaf was seen to consist of an electron-translucent layer (the so-called “cuticle proper”; Fig. 3e-h, black arrowhead), and an electron-dense layer, (the cuticle layer; Fig. 3e-h, white arrowhead) in all three WT cultivar plants and the mutant, as reported by Chen et al. (2003). Interestingly, the cuticle layer on the adaxial surface of the leaf was apparently bolder in WT (Fig. 3e, 3f, 3g), compared with the *pe lg* mutant (Fig. 3h). In contrast, the electron densities of the cuticle layer on the abaxial surface were the same for WT plants and the *pe lg* mutant (Fig. 3i-l). These results suggested that the cuticle layer might be thinner in the *pe lg* mutant compared to WT only on the adaxial surface. Alternatively, it is possible that the cuticle components on the adaxial surface of the mutant leaf were somewhat altered.

Water permeability enhancement in the *pe lg* mutant

In general, *Arabidopsis* mutants with a thinner cuticular layer are more water permeable, most likely due to an increase in the permeability of the leaves in particular (Samuels 2008). If the cuticle layer of the *pe lg* mutant is thinner or distorted, we would expect the mutant leaves to be more water permeable, as has been reported previously (Xiao et al. 2004; Li et al. 2007). We therefore carried out aqueous TB staining tests as described previously, to evaluate the water permeability due to surface deficiency (Tanaka et al. 2004). In this experiment, the fifth leaf and fruit at the mature green stage were incubated

1 with TB solution for 5 min, and the staining intensities were compared. The abaxial sides were
2 similarly stained in WT and the mutant (data not shown). However, in the adaxial sides, we found that
3 WT leaf was barely stained (Fig. 4a), whereas the mutant leaf stained more quickly (Fig. 4b),
4 supporting the idea that the cuticular layer of the leaf on the adaxial side is thinner and that water
5 permeability of the leaves is greater in the *pe lg* mutant than in WT. Similarly, WT fruit was barely
6 stained (Fig. 4c) while the mutant fruit was well stained, in a spotty pattern (Fig. 4d). In the *pe lg*
7 mutant, TB dye actually penetrated into the outer surface of the fruits (Fig. 4e). These results showed
8 that the *pe lg* mutant had a higher water permeability than WT.

9
10 Transpiration experiments

11 Next we measured the transpiration rate of the leaves over a 4 h period because it is known that
12 disruption of the leaf cuticle layer leads to increased transpiration (Chen et al. 2003; Aharoni et al.
13 2004; Bessire et al. 2007). Consistent with our prediction, the *pe lg* mutant showed a significantly
14 higher transpiration rate than any of the three WT cultivars (Fig. 5a). Figure 5b shows the leaf
15 morphology after 4 h of transpiration assay; the *pe lg* leaf was more wilted compared with WT
16 cultivars. The transpiration rate of the fruits was also measured to evaluate water permeability of the
17 thinner cuticle layer of the *pe lg* mutant (Fig. 6). Contrary to our prediction, the transpiration rate of the
18 *pe lg* fruit was significantly lower than WT cultivars K091 and RC17 (Fig. 6a). After 6 days incubation,
19 the fruit surface of K091 and RC17 became wrinkled due to water loss (Fig. 6b). However, consistent
20 with the transpiration measurement, the fruit surface of KGM942 and Ec-1 was almost unchanged after
21 the experiment (Fig. 6b). The dry matter contents of the leaves and fruits were not significantly
22 different between the *pe lg* mutant and WT cultivars (Supplemental Fig. S4), suggesting that the water
23 content of these tissues did not differ between the *pe lg* mutant and WT cultivars. These results are
24 consistent with the fact that cuticular mutants with reduced amounts of cuticular components generally
25 exhibit enhanced water permeability and transpiration rates due to the thinner cuticle layer (Chen et al.
26 2003; Xiao et al. 2004).

27 We then examined the density of leaf stomata and trichomes on the abaxial surface of the leaves to
28 examine the possibility that the enhanced transpiration rate is due to a difference in leaf surface
29 structure (Supplemental Figs. S5 and S6). Although the stomatal density varied according to the genetic
30 makeup, even in WT cultivars, the *pe lg* mutant had fewer stomata on the adaxial surface of the leaf

compared to the two WT cultivars (K091, RC17) (Supplemental Fig. S5). In contrast, the stomatal density of the abaxial surface of the leaf did not differ significantly between the two WT cultivars (RC17, Ec-1) and the *pe lg* mutant. The density of trichomes did not differ among all the genotypes (Supplemental Fig. S6). These results suggest that the differences in transpiration rate resulted from water leakage from the thinner cuticle membranes of the leaf, rather than from the increased numbers of stomata.

Chlorophyll leaching assays

To investigate other cuticle properties, a chlorophyll leaching assay was performed. In this experiment, the leaves were immersed in organic solvent for 8 h, and the degree of chlorophyll leaching was determined. It is known that cuticular mutants show increased leaching (Aharoni et al. 2004; Schnurr et al. 2004; Curvers et al. 2010). In contrast to our prediction, we observed no significant difference in the chlorophyll leaching rates between WT and the *pe lg* mutant (Supplemental Fig. S7). To date, we have not been able to find any convincing reasons why the mutant did not show a higher rate of chlorophyll leaching. However, using an organic solvent represents a more aggressive treatment than using an aqueous solvent, and this may interfere more strongly with plant tissues. Thus, it is possible that the leaf epidermis of the *pe lg* mutant is partially distorted, making it more permeable to water, but that it exhibits the same properties as WT when treated with an organic solvent.

Metabolite fingerprinting of cutin and wax fractions

The cutin and the wax composition at the ripe red stage was estimated by metabolite fingerprinting. Supplemental Fig. S8a-d show typical chromatograms obtained from the cutin extracts. Overall total ion chromatograms (TICs) of cutin extracts were similar between WT lines (Supplemental Fig. S8a, S8b, S8c). On the other hand, the TIC of the cutin extracts of *pe lg* mutant showed that the peaks detected at the region from retention index at 2,000 to retention index at 2,600, where contains a peak identified as 16-hydroxyhexadecanoic acid, were dramatically decreased (Supplemental Fig. S8d). Although a part of metabolites were identified or annotated (Supplemental Table 1), we could not conduct complete identification of these peaks because we could not purchase some of the chemical standards such as 10,16-dihydroxypalmitate. Further extensive analysis of cutin component will be required to elucidate aberrant cuticle formation of the *pe lg* mutant.

1 The TICs obtained from wax extracts are shown in Supplemental Fig. S9. Unlike in the case of the
2 fingerprint of the cutin fraction, the fingerprint of the wax fraction looked similar among these cultivars.
3 This phenomena is also observed in the cutin mutants in tomato (Isaacson et al. 2009), supporting that
4 the *pe lg* mutation is probably related to cutin formation.
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Discussion

Thin cuticle layer is formed in *pe lg* fruit

In this study, the cytological and physiological properties of the *pe lg* epidermis structure were investigated. It appears that the fruit cuticle of the *pe lg* mutant was thinner than that of WT (Fig. 1e-j) and contained less cutin components than WT (Supplemental Fig. S8). Furthermore, it is likely that the accumulation of flavonoids such as naringenin is suppressed in the *pe lg* mutant fruit epidermis (Fig. 1c, 1d, Supplemental Fig. S2). Since the *pe lg* mutant produced an extremely thin fruit cuticle layer, the absence of flavonoids could be due to a failure in flavonoid accumulation in the cuticle, where pigment is accumulated (Hunt and Baker 1980). To date, an absence of flavonoids from the fruit epidermis has only been reported in the *y* and *dfd* mutants (Saladié et al. 2007; Adato et al. 2009; Ballester et al. 2010). However, unlike the *pe lg* mutant, these two mutants do not show reduced cuticular formation. In addition, a previous genetic study has shown that the *pe* mutation is epistatic to the *y* mutation (Kerr 1982). Therefore, it is most likely that the *pe lg* mutant is distinct from these mutants and that *pe lg* should be recognized as a novel type of mutation affecting flavonoid biosynthesis in fruit.

The *lecer6* mutant lacks C31 alkane in its fruit waxes, resulting in increased water permeability, and the *lecer6* fruit finally becomes wrinkled at the mature ripe red stage (Vogg et al. 2004; Leide et al. 2007). This suggests that long chain alkanes in fruit waxes are critical for water permeability and for maintaining adequate transpiration in fruit. Other tomato mutants showing defective fruit cuticle formation have been reported, such as the three *cd* mutants (*cd1-3*) which have thinner cuticle layers, similar to the *pe lg* phenotype (Isaacson et al. 2009). However, it has been reported that these *cd* mutations affect only the fruit cuticle layer, but not the leaf cuticle layer, unlike the *pe lg* mutant. Despite the thinner cuticle layer, the water transpiration rate of the *cd2* fruit is similar to that of WT. Biochemical analysis has shown that total cutin acid monomer levels in fruits of the *cd* mutants are dramatically reduced, whereas total wax loads are not affected compared to the corresponding WT cultivar M82. These results suggest that both wax and polymeric cutin play important roles in water permeability and shelf-life, and that complex physical interactions between wax and cutin, or other physical or biochemical factors, might be involved in control of fruit epidermis permeability. Unlike the *lecer6* mutant, the *pe lg* mutant fruits did not become wrinkled at the ripe red stage (Fig. 1b), indicating that resistance to water dehydration is sustained in spite of the thinner cuticle layer, whereas water permeability of the leaf was enhanced (Fig. 6a, 6b). In contrast, the TB test showed enhanced

1 water permeability in the *pe lg* mutant fruit (Fig. 4d). It is possible that water transpiration rates may
2 differ between the two sides of the fruit surface. In fact, it seems likely that water invaded
3 progressively from outside (Fig. 4e), whereas there is less water transmission from the inside of the *pe*
4 *lg* fruit surface (Fig. 6). Further research is needed to clarify this issue.

5
6 Leaf cuticle is also disrupted in the *pe lg* mutant

7 The cuticle properties of leaves were examined in WT cultivars and the *pe lg* mutant using TEM. The
8 electron density of the cuticle layer in the mutant was lower compared with that of WT plants (Fig. 3h).
9 Similar to the *pe lg* mutant, TEM observations have often shown reduced electron density of the cuticle
10 layer in *lacs2*, *gpat4/8*, *wax2*, and *cyp86a2* mutants of *Arabidopsis* (Chen et al. 2003; Schnurr et al.
11 2004; Xiao et al. 2004; Li et al. 2007). Among these, only the *lacs2* mutant has a reduced cuticle layer
12 on the abaxial surface of the leaf, but not on the adaxial surface. A defect in the leaf cuticle was also
13 observed in the *pe lg* mutant, but only on the adaxial surface (Fig. 3h), whereas cuticle formation on the
14 abaxial surface was similar to that of WT (Fig. 3i). However, observation of the abaxial surface of the
15 leaf revealed that the shapes of guard cells and epidermal cells were deformed on this surface (Fig. 2c,
16 2d), as was also observed in the *lacs2* mutant in *Arabidopsis* (Schnurr et al. 2004), indicating that the
17 *pe lg* mutations affect both surfaces of the leaf structure. It is possible that the *pe lg* mutations directly
18 affect epidermal structure as well as cuticular structure.

19 Changes in physiological properties, such as increased water permeability (Fig. 4) or enhanced
20 transpiration rates (Fig. 5), have also been observed among the *gpat4/gpat8*, *lacs2*, *cyp86a2*, and *pe lg*
21 mutants. It is therefore most likely that the decreased barrier properties in the *pe lg* mutant resulted
22 from disorders in the cuticle (Figs. 1 and 3). It is also possible that changes in the barrier properties
23 were in part due to disorders in the epidermal structure (Fig. 2).

24 While previous report observed enhanced chlorophyll leaching in the *lacs2* mutant when treated with
25 an organic solvent, our experiment showed no difference between WT and the *pe lg* mutant
26 (Supplemental Fig. S7). These conflicting results suggest that the effects of the tomato *pe lg* mutation
27 on cuticular formation and cellular leaf structure are not totally consistent with those observed in
28 *Arabidopsis* cuticular-related mutations. Taken together with the fact that the *pe lg* mutant is
29 phenotypically different from previously reported tomato cuticle mutants (i.e. *lecer6* mutant, *dfd*
30 mutant, and the three *cd* mutants), we suggest that the *pe lg* mutant should be placed into a novel class

1 of tomato cuticle-defective mutants.

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3

4 Acknowledgement

5 We thank prof. Endo TR (Laboratory of Plant Genetics, Graduate School of Agriculture,
6 Kyoto University, Kyoto, Japan) for revising the manuscript.

7

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Figure legends

Fig. 1 Comparison of fruit phenotypes in WT and *pe lg* mutant. Ripe red stage of WT (RC17) fruit (**a**) and *pe lg* (KGM942) fruit (**b**). Epidermis detached from WT fruit (**c**) and *pe lg* ripe red fruit (**d**). Microscopic images of the fruit surface of WT (**e**) and *pe lg* (**f**). SEM images of epidermal sections of WT fruit (**g**) and *pe lg* fruit (**h**). Microscopic images of epidermal sections of WT fruit (**i**) and *pe lg* fruit (**j**). Arrowheads and arrows indicate epidermal cells and cuticle membrane, respectively. Scale bars = 1 cm (**a-d**), 50 μ m (**e-j**)

Fig. 2 Comparison of leaf phenotypes in WT (RC17) and *pe lg* mutant. Photographic images of the fifth leaf in WT (**a**) and *pe lg* (**b**). SEM images of leaf surface of WT (**c**) and *pe lg* (**d**). Insets indicate magnified images of stomata. Scale bars = 1cm (**a-b**), 20 μ m (**c-d**)

Fig. 3 Leaf cross-sections of WT and *pe lg* mutant. **a-d** Leaf cross-sections observed by light microscopy. **e-l** TEM images of the adaxial surface (**e-h**) and the abaxial surface (**i-l**). K091 (WT, **a**, **e**, **i**); RC17 (WT, **b**, **f**, **j**); Ec-1 (WT, **c**, **g**, **k**); KGM942 (*pe lg*, **d**, **h**, **l**). The cuticle proper is indicated by black arrowheads and the cuticle layer is indicated by white arrowheads (**e-l**). Scale bars = 50 μ m (**a-d**), 500 nm (**e-l**)

Fig. 4 Water permeability of the leaf and fruit cuticle. TB staining pattern of WT cultivar RC17 (**a**, **c**) and *pe lg* (**b**, **d**). Inset shows a magnified image of a TB stained spot (**e**). Scale bars = 1 cm (**a-d**), 50 μ m (**e**)

Fig. 5 Comparison of transpiration rates of the leaves between WT and *pe lg* mutant. **a** Leaf transpiration rate of three wild-type cultivars (K091, RC17, Ec-1) and the *pe lg* cultivar (KGM942). Vertical bars indicate SD ($n=5$). Differently lettered columns indicate significant differences. **b** Intact leaves of three wild type cultivars and *pe lg* cultivar. Scale bar = 1 cm

Fig. 6 Comparison of water permeability of ripe red fruit between WT and *pe lg* mutant. **a** Fruit transpiration rate of three WT cultivars (K091, RC17, Ec-1) and the *pe lg* cultivar (KGM942). Vertical bars indicate SD ($n=5$). Differently lettered columns indicate significant differences. **b** Fruits of three wild type cultivars and the *pe lg* cultivar. Scale bar = 1 cm

Table 1 Segregation test for the *pe* and the *lg* phenotypes
Fruits were picked at the red ripe stage. *pe* fruits were determined by their appearance. Similarly, *lg* leaves were determined by observing appearance of mature leaves

Phenotype	<i>N</i>	Probability
<i>PE LG</i>	252	0.754
<i>pe LG</i>	0	0.000
<i>PE lg</i>	0	0.000
<i>pe lg</i>	82	0.246
Total	334	
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χ^2 -value for single gene, 3:1 ratio	3.2E-05	
Probability of χ^2	1.00	

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Comparison of fruit phenotypes in WT and *pe lg* mutant.
Ripe red stage of WT (RC17) fruit (**a**) and *pe lg* (KGM942) fruit (**b**). Epidermis detached from WT fruit (**c**) and *pe lg* ripe red fruit (**d**). Microscopic images of the fruit surface of WT (**e**) and *pe lg* (**f**). SEM images of epidermal sections of WT fruit (**g**) and *pe lg* fruit (**h**). Microscopic images of epidermal sections of WT fruit (**i**) and *pe lg* fruit (**j**). Arrowheads and arrows indicate epidermal cells and cuticle membrane, respectively.
Scale bars = 1 cm (**a-d**), 50 μm (**e-j**)
83x147mm (300 x 300 DPI)

Comparison of leaf phenotypes in WT (RC17) and *pe lg* mutant.
Photographic images of the fifth leaf in WT (**a**) and *pe lg* (**b**). SEM images of leaf surface of WT (**c**) and *pe lg* (**d**). Insets indicate magnified images of stomata. Scale bars = 1cm (**a-b**), 20 μ m (**c-d**)
83x57mm (300 x 300 DPI)

Leaf cross-sections of WT and *pe lg* mutant.
a-d Leaf cross-sections observed by light microscopy. **e-l** TEM images of the adaxial surface (**e-h**) and the abaxial surface (**i-l**). K091 (WT, **a, e, i**); RC17 (WT, **b, f, j**); Ec-1 (WT, **c, g, k**); KGM942 (*pe lg*, **d, h, l**). The cuticle proper is indicated by black arrowheads and the cuticle layer is indicated by white arrowheads (**e-l**). Scale bars = 50 μ m (**a-d**), 500 nm (**e-l**)
173x109mm (300 x 300 DPI)

Water permeability of the leaf and fruit cuticle.
TB staining pattern of WT cultivar RC17 (**a**, **c**) and *pe lg* (**b**, **d**). Inset shows a magnified image of a TB stained spot (**e**). Scale bars = 1 cm (**a-d**), 50 μ m (**e**)
83x139mm (300 x 300 DPI)

Comparison of transpiration rates of the leaves between WT and *pe lg* mutant.
a Leaf transpiration rate of three wild-type cultivars (K091, RC17, Ec-1) and the *pe lg* cultivar (KGM942). Vertical bars indicate SD (N=5). Differently lettered columns indicate significant differences. **b** Intact leaves of three wild type cultivars and *pe lg* cultivar. Scale bar = 1 cm
83x199mm (300 x 300 DPI)

Comparison of water permeability of ripe red fruit between WT and *pe lg* mutant.
a Fruit transpiration rate of three WT cultivars (K091, RC17, Ec-1) and the *pe lg* cultivar (KGM942). Vertical bars indicate SD (N=5). Differently lettered columns indicate significant differences. **b** Fruits of three wild type cultivars and the *pe lg* cultivar. Scale bar = 1 cm
83x190mm (300 x 300 DPI)

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Title:

A novel class of *sticky peel* and *light green* mutations causes cuticle deficiency in leaves and fruits of tomato (*Solanum lycopersicum*)

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Supplemental Fig. S1 Fresh weight and size of leaflet and fruit of three WT cultivars and the *pe lg* mutant. **a** Fresh weight of the leaflets. **b** Fresh weight of the fruits. **c** The leaflet length and the fruit diameter. Three WT cultivars K091, RC17, and Ec-1, were used as controls. Breeding line KGM942 carries *pe lg* mutations. Vertical bars indicate SD ($n=3$). Differently lettered columns indicate significant differences

Supplemental Fig. S2 Naringenin accumulation in ripe red fruits of WT and *pe lg* mutant.

Vertical bars indicate SD ($n=3$). Differently lettered columns indicate significant differences

Supplemental Fig. S3 Chlorophyll contents of WT and *pe lg* mutant. Vertical bars indicate SD ($n=5$). Differently lettered columns indicate significant differences

Supplemental Fig. S4 Dry matter contents of mature leaves and ripe red fruits in the WT and *pe lg* mutant. Vertical bars indicate SD ($n=5$). Differently lettered columns indicate significant differences

Supplemental Fig. S5 Stomatal density of WT and *pe lg* mutant. Vertical bars indicate SD ($n=5$). Differently lettered columns indicate significant differences

Supplemental Fig. S6 Trichome density of WT and *pe lg* mutant. Vertical bars indicate SD ($n=5$). Differently lettered columns indicate significant differences

Supplemental Fig. S7 Chlorophyll leaching assay using leaves of the WT and *pe lg* mutant.

The leaves of WT (RC17) and *pe lg* mutant were treated for 1-8 h with 80% EtOH. Vertical bars indicate SD ($n=5$). Differently lettered columns indicate significant differences

Supplemental Fig. S9 Total ion chromatograms (TICs) of cuticular wax extracts of Ec-1 (a), K091 (b), RC17 (c) and KGM942 (d)

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Supplemental Table 1 List of identified or annotated metbolites

Metabolite	Retention Index	Identified (I) or Annotated (A)
n-Tritriacontane	3300.0	I
Triacontane	2999.4	I
alpha-Tocopherol	3152.4	I
D-Sucrose	2613.0	I
Oleate	2205.0-2227.0	I
n-Octadecanoic acid	2236.0	I
Linoleic acid	2208.0	I
n-Nonacosane	2900.1	I
n-Hentriacontane	3100.2	I
n-Hexadecanoic acid	2039.2	I
n-Dotriacontane	3199.8	I
Citric acid	1800.4	I
16-Hydroxyhexadecanoic acid	2379.5	I
π Eudesmol	1765.3	A
n-Tetradecanoic acid methyl ester	1721.5	A
n-Tetracosanoic acid	2828.7	A
n-Tetracosanoic acid methyl ester	2729.5	A
Stigmasterol	3316.8	A
beta-Sitosterol	3382.1	A
Phosphoric acid	1261.6	A
4-Oxo-pentanoic acid, ethyl ester	1061.5	A
Mannose, 6-deoxy-	1608.6	A
Hexanoic acid	1070.5	A
16-hydroxy-hexadecanoic acid, methyl ester	2272.9	A
n-Hexadecanoic acid methyl ester	1924.3	A
n-Heptadecanoic acid methyl ester	2024.9	A
Glycine	1227.9	A
1,6-Anhydro-beta-D-Glucose	1693.6	A
1-O-methyl-, alpha-D-Glucopyranoside	1864.9	A
Ethyl Laurate	1990.5	A
n-Eicosanoic acid methyl ester	2326.5	A
Linolenic acid methyl ester	2107.3	A
7-Hexadecenoic acid methyl ester	1904.5	A
Eremophil-1(10)-ene	1707.4, 1750.2	A
1-Propene-1,2,3-tricarboxylic acid, trimethyl ester	1437.7	A
Trimethyl 2-hydroxy-1,2,3-propanetricarboxylic acid	1540.4	A