Running title: tomato fruit brightness mutants

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Analyses of tomato fruit brightness mutants uncover both cutin-deficient and cutin-abundant mutants and a new hypomorphic allele of GDSL lipase

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Abstract

Cuticle is a protective layer synthesized by epidermal cells of the plants and consisting of cutin covered and filled by waxes. In tomato fruit, the thick cuticle embedding epidermal cells has crucial roles in the control of pathogens, water loss, cracking, postharvest shelf-life and brightness. To identify tomato mutants with modified cuticle composition and architecture and to further decipher the relationships between fruit brightness and cuticle in tomato, we screened an EMS (ethyl methanesulfonate) mutant collection in the miniature tomato cultivar Micro-Tom for mutants with altered fruit brightness. Our screen resulted in the isolation of 16 glossy and 8 dull mutants displaying changes in the amount and/or composition of wax and cutin, cuticle thickness and surface aspect of the fruit as characterized by optical and Environmental Scanning Electron microscopy. Main conclusions on the relationships between fruit brightness and cuticle features are that (i) screening for fruit brightness is an effective way to identify tomato cuticle mutants, (ii) fruit brightness is independent from wax load variations, (iii) glossy mutants show either reduced or increased cutin load, (iv) dull mutants display alterations in epidermal cell number and shape. Cuticle composition analyses further allowed the identification of groups of mutant displaying remarkable cuticle changes e.g., mutants with increased dicarboxylic acids in cutin. Using genetic mapping of a strong cutin-deficient mutation, we next discovered a novel hypomorphic allele of GDSL lipase carrying a splice junction mutation, thus highlighting the potential of tomato brightness mutants for advancing our understanding of cuticle formation in plants.

Key words: tomato, fruit, cuticle, mutant, EMS, brightness, cutin, wax, GDSL lipase

Abbreviations: EMS, ethyl methanesulfonate; ESEM, Environmental Scanning Electron Microscopy; FW, fresh weight
INTRODUCTION

The epidermis of all aerial plant organs is covered with an extracellular layer, the cuticle, which is synthesized by the epidermal cells. Cuticle is localized on the outer face of primary cell walls and is largely composed of cutin embedded with polysaccharides, filled with intracuticular waxes and covered with a thin layer of epicuticular waxes (Nawrath, 2006). Cutin is a polyester of glycerol, hydroxy and epoxy fatty acids; in most species, the main cutin monomers are C16 and C18 ω-hydroxy fatty acids (Pollard et al., 2008). Besides cutin, another lipid polyester named suberin typically contains in addition α,ω-dicarboxylic acids, hydroxycinnamic acids and fatty alcohols. Suberin forms a hydrophobic layer in cell walls of specific plant organs (e.g., roots and seeds) or is synthesized in response to stress (Pollard et al., 2008). Waxes are a mixture of very long chain fatty acids (VLCFA, C24-C34), and their derivatives such as alkanes, aldehydes, primary and secondary alcohols, ketones or esters and include occasionally of triterpenoids and phenylpropanoids (Kunst and Samuels, 2009).

In the recent years, availability of Arabidopsis genome sequence, high throughput gene expression analysis tools and mutant collections enabled deciphering the biosynthetic pathways and transport networks involved in cutin, suberin and wax biosynthesis (Pollard et al., 2008; Li-Beisson et al., 2009; Yeats and Rose, 2013). The synthesis of cutin monomer starts with the synthesis of long chain fatty acids in the plastids. Fatty acids are then transported to the cytoplasm where they undergo a series of modifications including the activation to coenzyme A thioesters by long-chain acyl-CoA synthetases (LACS), oxidation by cytochrome P450-dependent fatty acid oxidases (CYP) and esterification to glycerol-based acceptor by glycerol-3-phosphate acyl transferases (GPAT) to produce acyl-glycerols (Li-Beisson et al., 2009; Pollard et al., 2008). Though the sequential order of the reactions remains to be determined, the implication in cutin biosynthesis of several LACS, CYP86A and CYP77A and GPAT has been confirmed in Arabidopsis. Mechanisms of transport of the cutin monomers and their assembly into the cuticle remain largely unknown. The plasma membrane ATP-binding cassette (ABC) transporters have been implicated in the transport of both wax and cutin to the apoplast while lipid transfer proteins (LTP) very likely contribute to the transport of cutin monomers through the cell wall to the cutin layer (Yeats and Rose, 2013). The implication of enzymes of the GDSL lipase family in cutin assembly, suspected for a long time (Reina et al., 2007; Mintz-Oron et al., 2008), has been demonstrated very recently in tomato (Girard et al., 2012; Yeats et al., 2012).

Molecular genetic studies were also of considerable help in the recent identification of key proteins in suberin and wax biosynthesis. Same gene families as for cutin are likely
contributing to suberin formation (Franke et al., 2012), which includes in addition specific steps such as fatty acid elongation involving β-keto acyl-CoA synthases (KCS) and primary alcohol synthesis implicating fatty acyl reductases (FAR; Domergue et al., 2010). Likewise, several major enzymes of wax biosynthesis have been identified only very recently (Bernard and Joubès, 2013). Wax biosynthesis involves several steps including the synthesis of VLCFA through a multi enzyme fatty acid elongase complex and the synthesis of VLCFA derivatives through either the alcohol forming pathway, which gives rise to primary alcohols and wax esters, or the alkane forming pathway producing aldehydes, alkanes, secondary alcohols and ketones. Export of wax through plasma membrane occurs via ABC transporters and glycosylphosphatidylinositol-anchored LTP (LTPG) (Kunst and Samuels, 2009; Yeats and Rose, 2013).

Coordinated regulation of metabolic pathways controlling cuticle formation has been demonstrated for transcription factors of the SHINE/WIN (SHN) family (Aharoni et al., 2004; Broun et al., 2004) and recently for MIXTA-like transcription factors (Oshima et al., 2013). Several of their downstream targets involved in cutin synthesis, modification and assembly, and beyond in cell wall formation and structure and in epidermal cell patterning (Shi et al., 2013), have been identified. Other transcription factors of the MYB protein family, HD-ZIP IV family and WW domain protein were shown to control cuticle formation and, for some of them, were suggested to take part of a regulatory network controlling epidermal cell patterning (Yeats and Rose, 2013).

Still, despite these advances and new technological developments, much remains to be known on the regulation, synthesis, assembly and structure of the cuticle and their influence on cuticle properties. The thick and easy-to-isolate peel from tomato has long been used for studying the biomechanical properties of plant cuticle (Dominguez et al., 2011). In the last years, the increased availability of tomato genomic resources, including saturated genetic linkage maps, markers and very recently tomato genomic sequence (Tomato Genome Consortium, 2012) offered unprecedented possibilities for exploiting tomato as a model for studying plant cuticle (Hovav et al., 2007; Adato et al., 2009; Isaacson et al., 2009; Girard et al., 2012; Yeats et al., 2012; Shi et al., 2013). To fully explore the relationships between cuticle composition and cuticle properties and to discover new genes and functions, new sources of genetic variability affecting tomato genes involved in wax, cutin and suberin synthesis and regulation are required.

In the presented study, we focused on tomato cuticle mutants identified through screening for fruit brightness (glossy or dull fruits) a tomato EMS mutant collection in the miniature cultivar Micro-Tom. Up to 24 tomato fruit cuticle mutants with modified fruit brightness but no otherwise major plant or fruit phenotypic changes were selected. Cytological and biochemical
analyses revealed that mutant fruit with altered brightness displayed wide variations in cutin load and composition and in epidermal patterning. Only few changes in wax composition and increases in wax load were observed. Fruit brightness modifications could not be attributed to a single cause but rather to combinations of various and sometimes opposite alterations of fruit cuticle. We further identified the mutation underlying a glossy cutin-deficient mutant by genetic mapping of the corresponding cuticle-associated traits and discovered a novel allele of the tomato GDSL lipase involved in cutin polymerization (Girard et al., 2012; Yeats et al., 2012). Thus our study highlights how the exploitation of artificially-induced genetic diversity and of genomic tools currently available in tomato can efficiently contribute to the study of cuticle biosynthesis and properties in plants.

RESULTS

Screening Micro-Tom EMS mutant collection for plants with altered fruit brightness

Analysis of cuticle formation and properties using reverse genetic approaches may be hampered by other modifications of plant and fruit physiology often induced in strong cuticle mutants. In this study, we chose to focus on mild tomato fruit cuticle mutants displaying no obvious phenotypic alterations such as dwarfism, wilting or organ fusion in plant or large cracks, multiple micro-cracks (russetting) and strong water loss in fruit. Towards this end, the criterion we used for selecting tomato mutants was fruit brightness, the variation of which results from milder modification of fruit surface. In addition, thin cuticle and glossy fruit appearance associated with adequate postharvest shelf-life and absence of fruit defects are among the desirable breeding traits for several types of fresh-market tomatoes.

The tomato mutant collection used was an EMS (ethyl methanesulfonate) mutant population generated in the miniature cultivar Micro-Tom by our group (Causse and Rothan 2007; Just et al., 2013). The collection comprises ca 3500 highly-mutagenized mutant families thoroughly phenotyped (12 plants per M2 families) for ca 150 plant and fruit phenotypic criteria ranged in categories and sub-categories. All the phenotypic data are compiled and stored into a dedicated web-searchable database called MMDB (MicroTom Mutant DataBase). Selection of fruit brightness mutants was done using requests centered on the fruit (category: fruit; sub-categories: color, epidermis, brightness i.e. fruit glossier or duller than the wild-type control). Strong fruit cuticle/color mutants such as the slyp86a9 cuticle synthesis mutant recently identified from the same Micro-Tom mutant collection (Shi et al., 2013), were excluded from the analysis. As shown in Figure 1, the first query using these criteria resulted in the identification of 274 mutant families. Taking into account additional
criteria such as the presence/absence of other gross plant and fruit phenotypic alterations and the previous confirmation (or not) of the observed fruit brightness phenotypes in independent cultures of the retained M2 families allowed the final selection of 40 mutant families.

For each selected family, 24 plants were grown in greenhouse. Visual evaluation of fruit brightness was done for each plant of each family on fruits at Red Ripe (RR) stage, in comparison with wild-type (WT) fruits (Supplemental Table S1). In the M2 families studied, which segregates for the EMS-induced mutations, a plant was considered as a fruit brightness mutant when all the fruits on that plant displayed the glossy or dull trait. Previous fruit brightness phenotypic annotation (dull or glossy fruit) was confirmed for 20 families (i.e., at least one plant in the family showing uniformly dull or glossy fruits) whereas 7 families displayed fruit brightness phenotypes opposite to those previously observed. In addition, two displayed both dull/glossy fruits on the same plants and 11 did not show any visual difference with the WT fruits. We finally selected the 20 confirmed mutant families plus 4 families displaying fruit brightness phenotypes opposite to those previously observed (i.e. one plant with dull or glossy fruits for each of the selected families) Among these 24 fruit brightness mutant plants, 16 were glossy mutants and 8 dull (Supplemental Fig. S1).

Cuticle is altered in fruit brightness mutants which display remarkable changes in cutin load and composition

In order to investigate whether variations in fruit brightness could be associated with quantitative and/or qualitative differences of cuticle, we analyzed wax and cutin load and composition of RR fruits (~45 days post-anthesis or DPA) collected on the 24 selected mutant lines.

Both the glossy and the dull mutants exhibited a wide range of variation in wax (identified compounds) load (Fig. 2 and Table I). For up to 50% of the mutants, no significant differences in cuticular wax load were observed relative to wild type (Fig. 2). Remarkably, the glossy and dull mutants displaying wax load variation all showed an increase in wax load in comparison with WT. However, while the glossy mutants showed a gradual variation in wax load from ~6 µg/cm² (i.e., close to WT values) to 13,5 µg/cm², most of the dull mutants had similar wax loads (7,6-9 µg/cm²) with the exception of one (P6D6 mutant; 13,1 µg/cm²).

When taking into account the unidentified wax compounds, which represent 17% of the total wax in the wild type and up to 40% in the P4E2 mutant, similar variations were observed (data not shown).

As shown in Table I, the compounds identified in the chloroform-soluble wax from the Micro-Tom cultivar are mainly constituted by alkanes, which represent more than 60% of the wax,
triterpenoids (α, β and δ type amyrin; ~18%), isoalkanes (7-8%) and alcohols (~3-4%). While changes in alkane and in wax load showed the same trend in most mutants, several mutants showed remarkable modifications of wax composition. Amyrins increased to ~29 to 33% of total wax load in the P17F12 and P23F12 glossy mutants and to almost ~30% in the P6D6 dull mutant. Alcohols increased to ~7 to 12% in the P11H2 and P4E2 glossy mutants. In conclusion, most fruit brightness mutants displayed no or slight variations in wax load and glossy mutants could not be distinguished from dull mutants based only on single wax analysis.

Wider range of variation was observed for cutin (identified compounds) load among the mutants (Fig. 3 and Table II). Again, as for wax load, the dull mutants displayed much less variations than the glossy mutants, the larger variations in cutin load in these mutants being a 60% increase (P16H5) relative to the WT. At the opposite, glossy mutants exhibited huge disparities between the most extreme phenotypes (Supplemental Fig. S2). Both strong reductions (down to 15% of WT) and increases (up to 220% of WT) in cutin load were observed, unlike wax load. The residual cutin load of the three most affected glossy mutants were 84.1 µg/cm² (P15C12), 162.6 µg/cm² (P23F12), 285 µg/cm² (P30B6) i.e. much below the WT (586.1 µg/cm²). In contrast up to five glossy mutants displayed significant increases in cutin load which ranged from ~ 722.9 µg/cm² (P4B6) to 1175.5 µg/cm² (P17F12). As in other tomato cultivars described to date (Girard et al., 2012; Mintz-Oron et al., 2008; Nadakuduti et al., 2012), cutin monomers are mostly constituted by polyhydroxy fatty acids (65%, with 9,16-dihydroxy-palmitic acid representing 95% of the total), ω-hydroxy fatty acids (11%) and in lesser amounts by fatty acids (~6%) and dicarboxylic acids (~5%) (Table II). While changes in cutin load were usually paralleled by similar changes in the various classes of cutin monomers, several glossy mutants displayed remarkable and specific patterns of accumulation of cutin monomers. Indeed, further exploration of the cutin composition data by Principal Component Analysis (PCA) and Hierarchical Clustering Analysis (HCA) revealed groups of mutants displaying similar cutin composition changes (Fig. 4). The first principal component (PC1) explaining 53% of the total variability, clearly separated three clusters on the positive side and one cluster on the negative side. The second principal component (PC2) explaining 23% of the total variability separated two clusters on the positive side and two clusters on the negative side. A bulk of 8 mutants, either dull or glossy, was not separated by the principal components PC1 and PC2 and were aggregated within the WT cluster.

Examination of PC1 and PC2 loadings (Supplemental Fig. S3) allowed the identification of cutin monomers responsible for cutin modifications in the various mutants. On the positive side of PC1 and PC2, the primary alcohols and 2-hydroxy fatty acids and the dicarboxylic
acids discriminated the P17F12 glossy mutant and the P5E1/P26E8 cluster of glossy mutants. The P17F12 mutant, in which the total cutin load is increased by more than twofold, exhibited a 30-fold enhancement of 2-hydroxy fatty acids, which increased from trace amounts in the WT (6.6 µg/cm²) to ~16% of the total cutin monomers in the mutant (~201 µg/cm²). The dicarboxylic acid contents were strikingly similar between the P5E1 and P26E8 mutants and increased from ~32 µg/cm² in the WT to 134-139 µg/cm² in the mutants. Another remarkable cluster groups the strong cutin-deficient mutants which are similarly affected for all cutin monomers (e.g., the P15C12 and the P23F12 mutants clustered on the negative side of PC1). On the positive side of PC1 and negative side of PC2, the poly hydroxy fatty acids, the ω-hydroxy fatty acids, the fatty acids and the cinnamic acid compounds were discriminating the P30A12/P18H8 cluster of glossy mutants. These mutants accumulate large amount of cutin monomers (X 1.6), like P17F12, but show a different composition, especially with respect to the 2-hydroxy fatty acids which amount is close to that of the WT (Table II). Last, a cluster discriminated on negative side of PC2 groups several dull and glossy mutants with moderate to large changes in cutin load.

Thus, though cutin is altered in most dull and glossy mutants, no obvious link could be made between fruit cutin load and/or composition and fruit brightness, as already observed for waxes. Moreover, glossy mutants can show either strong deficiencies (e.g., P15C12) or increases (e.g., P17F12) in cutin load (Fig. 3). To further explore the possible effect of wax and cutin co-variations on fruit brightness, we combined the biochemical (wax and cutin composition and load) and phenotypic data and analyzed them by HCA (Supplemental Fig. S4 and S5). However, as for cutin alone, no obvious relationships could be found between cutin and wax variations and the dull or glossy aspect of the fruit.

*Epidermal and Cuticle architecture affect fruit brightness*

In order to get more insights into the relationships between fruit brightness and cuticle, we selected three groups of mutants according to cutin monomer and/or wax load of the fruits and further characterized them by optical and scanning electron microscopy. Among these were glossy mutants with high cutin load (P18H8 and P17F12) and low cutin load (P23F12 and P15C12) and dull mutants with either high cutin load (P16H5) or high wax load (P6D6) (Fig. 5A). As expected, glossy mutants with low cutin load exhibited very thin cuticles when compared to WT, with cuticle thickness reduced by ~49% for P23F12 and ~67% for P15C12 (Fig. 5D and E). Surprisingly, cuticle thickness was very similar between WT fruits and glossy mutants with high cutin loads, despite the strong difference in cutin load between these mutants and WT (60 to 120% more cutin in P18H8 and P17F12 respectively). Examination of exocarp sections indicated that neither tissue structure nor epidermal cell size and shape were affected in these glossy mutants (data not shown). Dull mutants did not show any
significant difference in cuticle thickness relative to WT but both high cutin and high wax dull mutants displayed distinctive morphological alterations of the cuticle-encased epidermal cells, which appeared less elongated and more conical-shaped than in WT and other mutants.

When examined under environmental scanning electron microscopy (ESEM), native fruit surface from glossy mutants with low cutin load mutants looked much smoother than that of WT fruits, which showed more irregular surface with small domes (Fig. 5B). At the opposite, fruit surface from dull mutants was rough and entirely covered with circular-shaped dome-like structures, in agreement with optical microscopy observations (Fig. 5D). These differences appeared even more clearly when epicuticular waxes were removed from cuticle by treatment with chloroform (Fig. 5C). Cutinized epidermal cell walls were clearly visible in the WT while the surface of high cutin load glossy mutants remained remarkably smooth with no (P18H8) or very few (P17F12) surface irregularities. In contrast, de-waxing low cutin load glossy mutants revealed very thin-walled epidermal cells underneath the cuticle proper, with no cutin deposit on top of these cells unlike WT. De-waxing did not change the surface aspect of the dull fruits, though the dome-like structures which correspond to epidermal cells (Fig. 5D) were even more apparent. Their quantification clearly indicates that fruit epidermal cell number is increased by 66% to 122% in the dull mutants (Supplemental Table S3).

Together these observations suggest that both the cuticle and the development of the epidermis are altered in dull fruit mutants. In contrast, only cuticle is affected in glossy fruit mutants and increased fruit brightness can be provoked by both deficiency and increased accumulation of cutin.

The cutin-composition mutants P5E1 and P26E8 show constitutive alteration of suberin biosynthesis in the fruit

To investigate the mutations underlying the variations in fruit brightness, we focused on the glossy mutants since dull mutations are likely pleiotropic and affect epidermis. We further focused on cutin mutants because cutin load is possibly the major factor controlling fruit brightness in tomato (Girard et al., 2012; Nadakuduti et al., 2012; Yeats et al., 2012), and the possible effect of wax changes on glossiness have already been well described in Arabidopsis (Chen et al., 2003; Aharoni et al., 2004; Bourdenx et al., 2011). One way to identify the possible origin of the mutation is to combine the wealth of information now available on cuticle biosynthesis and regulation with the data on fruit surface chemistry. Two of the cutin-accumulation mutants that clustered together in PCA-HCA (P5E1 and P26E8 lines; Fig. 4) showed unusual high dicarboxylic acid content (15-18% of total monomers). Cutin compositions of these mutants are strikingly similar (Table II and Supplemental Table S2), suggesting that they are allelic. In addition, close examination of the dicarboxylic acid
components of the P5E1 and P26E8 mutants (Supplemental Table S2) indicates that both display a 2.5 fold increase in C16:0 dicarboxylic acid and a 5 fold increase in C18:1 dicarboxylic acid when compared to WT. In most species, except Arabidopsis and other Brassicaceae, this composition is considered as indicative of suberin (Pollard et al., 2008). Remarkably, no russetting or any other visible mark of suberin accumulation had been observed in the fruit sample analyzed, which presented a uniform glossy appearance. Following cuticle composition analysis, we therefore planted one of these mutants (P26E8) in order to observe its cuticle phenotype. Close examination of the fruits revealed the obvious accumulation of suberin-like material at the distal end of the fruit (Supplemental Fig. S6), a trait not observed in the fruit sample previously analyzed. This result reinforces the hypothesis that the biosynthesis of both cutin and suberin are altered in these mutants. Since growth period and position of the fruit on the plant apparently influence the extent of accumulation of this suberin-like material, this is likely under the control of the environmental conditions.

The P15C12 cuticle mutant displays constitutive cutin-deficiency in the fruit

Two other obvious targets are the P15C12 and P17F12 cutin mutants, which are respectively strong cutin-deficient (cutin load: 84.1 µg/cm²) and strong cutin-accumulating (cutin load: 1175.5 µg/cm²) mutants. Fruits from the P17F12 mutant display obligatory parthenocarpy and plants carrying the cuticle mutation never set seeded fruits, despite attempts of pollination with WT pollen. This mutant was therefore not studied further. In contrast, the P15C12 mutant, in which plant and fruit were not affected by the mutation except for cuticle alteration, was further characterized along fruit development for cutin load and water loss (Fig. 6A- C). At 17 days post anthesis (DPA), fruits from WT and P15C12 mutant exhibited low cutin loads (between 30 and 50 µg/cm²). From then on, a striking accumulation of cutin associated occurred in the WT fruit during the next 8 days i.e. during the cell expansion phase of fruit growth. Cutin load reached ~615 µg/cm² at 25DPA in the WT and slightly increased thereafter until 695 µg/cm² at Red Ripe (RR) stage. At the opposite, cutin load of the P15C12 mutant continuously stayed at the same low level throughout fruit development until RR stage. Effect of the mutation on cutin accumulation in the cell walls from epidermal cells could be scored by analyzing the de-waxed fruit epidermis and measuring the thickness of the cutinized cell walls between two adjacent epidermal cells, thereafter called cutin width (Supplemental Fig. S7). The cutin width was almost twice more thinner in the P15C12 mutant than in the WT (Fig. 6D).

Alterations of cutin biosynthesis in the P15C12 mutant were also accompanied by large modifications of the cuticle permeability to water, though mutant wax load was 40% higher than that WT. The water loss kinetics of representative fruits from WT and P15C12 mutant
(Fig. 6B) clearly show the impaired ability of P15C12 detached fruits to avoid water loss, the mutant fruit retaining only 32.2% of its original weight at the end of the experiment versus 72.5% in the WT. Consistently, the tests of cuticle permeability performed on Mature Green (MG) fruit demonstrated that mutant fruit was very permeable to the toluidine blue (TB) dye, unlike WT fruit (Fig. 6C). Thus, besides fruit brightness, the mutation affecting fruit cutin load in the P15C12 mutant has dramatic consequences on the integrity and properties of its cuticle. Since these various phenotypic traits (fruit brightness, cutin width, water loss, permeability to TB) describe various aspects of the same mutation affecting cutin load, they were therefore used for characterizing tomato genotypes carrying the cuticle-deficiency allele found in P15C12.

**Mapping of the P15C12 locus and identification of the gds12-2 b mutation through candidate gene approach**

A F2 mapping population segregating for the cuticle-deficiency mutation found in P15C12 was generated through crossing the ‘Micro-Tom’ P15C12 homozygous mutant with a M82 dwarf mutant, previously selected amongst EMS mutants generated in the widely used tomato cultivar ‘M82’ (Menda et al., 2004). The rationales for this strategy are multiples (Just et al., 2013). First, by crossing Micro-Tom with a cultivar from the same tomato species (*Solanum lycopersicum*) and not with a wild tomato *e.g.* a *S. pimpinellifolium* accession, we avoid the segregation of multiple polymorphic traits, among which those affecting the cuticle (Yeats et al., 2012a). Second, the F2 mapping population of 110 plants can be grown in the greenhouse on a limited space (6 m² instead of 25 m² for *S. pimpinellifolium*). And last, a whole set of SNP markers polymorphic between ‘Micro-Tom’ and ‘M82’ has been recently developed (Shirasawa et al., 2010), allowing us to select 48 SNP markers evenly distributed along the 12 tomato chromosomes (Tomato Genome Consortium 2012). The mapping population was scored for cuticle-associated traits previously characterized in the P15C12 mutant: fruit brightness (3 classes, Supplemental Fig. S8A and B), cuticle permeability to TB dye (6 classes, Supplemental Fig. S8C and D), water loss (5 classes, Supplemental Fig. S8E and F) and cutin width (4 classes, Supplemental Fig. S8G and H). Frequency distributions show complex inheritance patterns for the various traits, probably revealing both the imprecisions in scoring the traits (fruit brightness, cuticle permeability) and the effects on cuticle properties of the interactions between cutin load and other cuticular traits which could segregate in the F2 population *e.g.* epidermal patterning or fruit surface chemistry. Nevertheless, the major locus controlling each trait was located in a 4.84 Mb region on chromosome 11, between markers 11289_715 and 10722_814 (Fig. 7A). LOD score, R² percentage and effect of QTL are presented in Table III.
We next screened the chromosomal region of interest for candidate genes with known implication in cutin synthesis or regulation. Among these were genes identified as specifically expressed in the outer epidermis of tomato by laser microdissection of various tomato pericarp tissues followed by RNAseq analysis (Matas et al., 2011). Two of them (Solyc11g007540 and Solyc11g006250; SGN: http://solgenomics.net/) were localized between the markers of interest on chromosome 11. The first presents a strong homology with an Arabidopsis gene (AT5GO4660.1) encoding a CYP77A4 cytochrome P450 oxidase catalyzing the epoxidation of free fatty acids (Sauveplane et al., 2009). No mutation in this gene was detected upon sequencing. The second encodes an acyltransferase of the GDSL esterase/lipase protein family recently shown to be involved in the extracellular deposition of cutin in tomato fruit (Girard et al., 2012; Yeats et al., 2012). The current locus name of Solyc11g006250 (1,004,368 to 1,006,899 nucleotides on chromosome 11) in the Solanaceae SGN database is GDSL2, while its given names were previously SIGDSL1 (Girard et al., 2012) and cutin synthase CD1 (Yeats et al., 2012). In agreement with SGN, the gene was herein named SIGDSL2.

Sequencing the SIGDSL2 (Solyc11g006250) gene from the WT and P15C12 plants revealed an A => T mutation disrupting the 3’ splice site of intron 4 (Fig. 7B). Since a mutated allele of SIGDSL2 was already described in tomato (Yeats et al., 2012), the P15C12 mutant is thereafter named gdsl2-b. As in other introns belonging to the major class of introns processed by the U2 spliceosome, the splice site sequences of intron 4 from SIGDSL2 gene fit the canonical GT-AG consensus borders, where the nearly invariant GT dinucleotide is at the 5’ end and the AG dinucleotide at the 3’ end of the intron. Point mutations in this 3’ AG dinucleotide, which is essential to the definition of the 3’ splice junction, may lead to the production of mRNA with unspliced intron 4. Actually, missplicing of intron 4 of SIGDSL2 leads to the accumulation of several species of SIGDSL2 mRNA in both the P15C12 mutant and its F1 hybrid (Fig. 7D). The larger mRNA effectively corresponds to unspliced mRNA, as confirmed by sequencing. In-frame reading of intron 4 produces 13 additional incorrect residues after exon 4 (Fig. 7C), thus leading to the production of a truncated protein in which the 64 residue C-terminal region is missing. Interestingly, a second mRNA is produced, which size is consistent with that of correctly spliced SIGDSL2 mRNA. However, close examination of the mutated sequence reveals the presence of a cryptic 3’ splicing site in exon 5, 17 nucleotides downstream of the canonical 3’ consensus splice sequence of intron 4, which is predicted to be favored in the mutant (Human Splice Finder: http://www.umd.be/HSF/HSF.html). Indeed, sequencing the smaller-sized mRNA confirmed the alternative splicing at this site. The new open reading frame in exon 5 leads to the synthesis of 8 new incorrect amino acids before truncation of the protein (Fig. 7C).
Real time quantitative PCR analysis of *SIGDSL2* expression in 20 DPA fruit revealed that accumulation of SIGDSL2 transcripts was reduced by ~96% in the P15C12 mutant when compared to WT (Fig. 7E). Immunoblot analysis of SIGDSL2 protein in 20 DPA fruit epidermis failed to detect the protein in the epidermis from the *gdsl2-b* fruit, in contrast to the WT fruit (Fig. 7F), suggesting that the *gdsl2-b* mutant carries a strong hypomorphic or null allele of the *SIGDSL2* gene, which is likely responsible for the cutin-deficiency phenotype observed in the P15C12 mutant line.
DISCUSSION

Considerable progress in the knowledge of cuticle synthesis and regulation have been made in the recent years thanks to the availability of *Arabidopsis* mutants and genomic tools. Main pathways for the synthesis of waxes and cuticle polymers have being deciphered (Pollard et al., 2008; Bernard and Joubès, 2013;) and increasing evidence on the mechanisms of transport of cuticular components and on the regulation of cuticle biosynthesis are now available (Yeats and Rose, 2013). Currently, one of the main challenges is to understand how cuticle properties are linked with cuticle composition and structure, how cuticle components interact with cell wall polymers, and how this will affect plant characteristics *e.g.* plant growth or resistance to biotic and abiotic stresses (Dominguez et al., 2011).

Though Arabidopsis remains the model of choice for plant functional genomics, this species is not well adapted for studying cuticle properties due to its very thin cuticle and some specificities (*e.g.* the high level of dicarboxylic acids in *Arabidopsis* cutin, unlike most other plants). In contrast, tomato fruit has a thick and easy-to-study cuticle synthesized along early fruit development (Yeats and Rose, 2013). Tomato has therefore long been used for studying cuticle biomechanics and permeability (Dominguez et al., 2011; Schreiber, 2010) and has recently emerged as a new model for functional genomics of cuticle formation in plants. Because tomato is both a major crop species and a model for fleshy fruits, a wealth of information and genomic tools are now available for this species (Tomato Genome Consortium, 2012). In addition, several major agronomical traits in tomato and in other fleshy fruit species *e.g.* fruit growth, visual aspect, cracking, water loss, resistance to pathogens and postharvest shelf-life are highly dependent on fruit cuticle (Bargel and Neinhuis, 2005; Saladie et al., 2007; Matas et al., 2009; Dominguez et al., 2011; Parsons et al., 2012). An increasing number of studies highlight the possibilities offered by tomato for analyzing cuticle architecture, mechanical properties and permeability (Lopez-Casado et al., 2007; Saladie et al., 2007; Mintz-Oron et al., 2008; Buda et al., 2009; Isaacson et al., 2009; Wang et al., 2011) and for discovering genes contributing to cuticle synthesis and regulation (Hovav et al., 2007; Mintz-Oron et al., 2008; Nadakuduti et al., 2012; Girard et al., 2012; Yeats et al., 2012; Shi et al., 2013). Nevertheless, to further our understanding of the relationships between cuticle composition and architecture and cuticle properties and performance in plants, new tomato cuticle mutants are highly needed (Dominguez et al., 2011).
Tomato EMS mutants for studying cuticle composition and properties

Collections of artificially-induced genetic diversity resulting from fast-neutron or Ethylmethane Sulfonate (EMS) mutagenesis are increasingly being used in tomato since generating saturated T-DNA collections remains out of reach in this species (Emmanuel and Levy, 2002). Though wild tomato species probably display larger variability in cuticle composition and architecture than cultivated tomato (Hovav et al., 2007; Yeats et al., 2012), EMS mutants provide several interesting features. The genetic background is almost identical for all mutant lines generated in a given cultivar, except for the induced point mutations, therefore allowing direct comparison between lines. Because point mutations induced by EMS are distributed at random over the whole genome, allelic series that include both loss-of-function and weak and strong hypomorphic alleles can be found for each target gene (Just et al., 2013). Tomato EMS mutant collections can be further exploited by TILLING, which allows the discovery of unknown point mutations in known candidate genes (Okabe et al., 2011), or through forward genetic approaches aiming at identifying the mutation underlying the mutant trait. Less straightforward than TILLING, this approach relies first on the phenotypic characterization of the mutant trait then on the identification of the mutation through map-based cloning, combined or not with candidate gene approach or, more recently, through whole genome sequencing (Abe et al., 2012; Just et al., 2013). Using this strategy, new cuticle-related genes involved in cuticle regulation (the cd2 HD-ZIP IV mutant; Isaacson et al., 2009), cutin monomer biosynthesis (the cyp86A9 mutant; Shi et al., 2013) and cutin polymerization (the cutin synthase mutant; Yeats et al., 2012) have recently been uncovered in tomato EMS mutant collections. However, when considering the large number of candidate genes involved in the regulation, synthesis and transport processes necessary for cuticle formation (Matas et al., 2011; Yeats and Rose, 2013), very few tomato cuticle mutants have been described to date (Nadakuduti et al., 2012; Kimbara et al., 2012; Shi et al., 2013).

In this study, screening the highly mutagenized EMS mutant collection of 3500 families generated in the miniature Micro-Tom cultivar (Just et al., 2013) revealed hundreds of mutants possibly affected in cuticle. Fruits from the selected mutants showed either strong fruit surface defects such as micro-cracks (russetting), cracks, strong shriveling, peel browning or less severe alterations such as increased water loss and altered fruit color or brightness (data not shown). Because strong cuticle alterations are detrimental to fruit quality, we preferred to focus in a first step on the phenotypic alterations only responsible for variations in fruit brightness (glossy or dull fruits), which likely arise from more subtle changes in cuticle properties. Pleiotropic mutations were also excluded since in that case the origin of the cuticular defect is not always easy to trace e.g., in fruit developmental mutants (Czerednik et al., 2012). The disadvantage of using fruit brightness as a screen for detecting
cuticle mutations is that this trait can be very sensitive to environmental conditions e.g. the growing season (data not shown). However, out of the 40 mutants examined (from the 274 originally found in the mutant database), more than 20 were further confirmed as being fruit brightness mutants. Among them, 8 displayed glossy/dull trait for both fruit and leaf (Supplemental table S1) indicating that fruit can be used as an attractive model for studying alterations of leaf cuticle.

Our study clearly shows that, though all selected brightness mutants displayed cuticle alterations, the glossy/dull fruit trait is not due to one single alteration. Recent studies of tomato mutants and transgenic lines established that increased fruit glossiness was associated with cutin deficiency (Isaacson et al., 2009; Girard et al., 2012; Nadakuduti et al., 2012; Shi et al., 2013). We indeed confirmed this relationship for several mutants (Fig. 4) but further showed that more complex cuticle architecture can be responsible for this trait. Most of the glossy mutants showed significant variations in either total cutin load or cutin composition (Table II). As shown in Supplemental Fig. S2 and summarized in Figure 8, both low and high cutin load mutants may exhibit increased fruit glossiness. Incident light will be either reflected in a mirror-like manner (specular reflection) or reflected in a broad range of directions (diffuse reflection) due to surface irregularities and light scattering e.g. by wax crystals (Pfündel et al., 2006). In WT tomato, cutin layer is thicker at the junction of the epidermal pavement cells (anticlinal pegs), thinner elsewhere (Fig.4D; Bargel and Neinhuis, 2005; Girard et al., 2012; Yeats et al., 2012), and therefore presents surface irregularities (Fig. 4B). The cutin layer is further covered by epicuticular waxes mostly constituted by aliphatic compounds, which form a film at the surface of the fruit (Vogg et al., 2004; Buschaus and Jetter, 2011). Due to these characteristics, WT fruit is moderately glossy. In high cutin load mutant, a thick cutin layer covers all cell surface and thus likely levels most irregularities resulting in a smooth surface, which can be seen in both native and de-waxed fruit (Fig. 4). This likely increases the specular reflection, hence giving a glossy aspect to the fruit. In contrast, the de-waxed surface of low cutin load mutants displays a very irregular aspect due to the presence of thin to very thin cutin layer. The epicuticular wax film covering these small surface irregularities likely “polishes” the surface of the fruit, which therefore displays increased glossiness. At the opposite, dull mutants present highly irregular surface due to an increased number of small epidermal cells of different morphological aspect, as shown by ESEM (Fig. 4). The rugged aspect of fruit surface, seen for both native and de-waxed fruit, is probably responsible for increased diffuse light reflection hence giving to the fruit a matt aspect (Fig. 8). Higher wax load of some mutants may also contribute to increase light scattering.
No glossy tomato fruit mutants with reduced total wax load were observed in the present study. In *Arabidopsis*, stem glossiness is generally indicative of altered epicuticular wax crystallization and is either due to general wax load reduction or to alteration of specific wax compounds (Jenks et al., 2002). Similar observations have been made in maize and in rice (Islam et al., 2009; Jenks et al., 2002). In addition, no organ fusion was observed among all the glossy/dull fruit mutants studied, in contrast to the glossy mutants described in *Arabidopsis* or to the *SICER6* loss-of-function wax tomato mutant (Smirnova et al. 2013). Though the participation of wax in the glossy aspect of the fruit cannot be excluded (several glossy mutants display significant increases in wax load), our results strengthen the conclusion that the glossy/dull fruit appearance in our tomato mutants is not due to wax deficiency. The most striking result is however that dull fruit aspect of tomato cuticle mutants appears primarily due to different epidermal cell differentiation. The relationship between epidermal cell differentiation and cuticle development is now well established and genes controlling both cell morphogenesis and cuticle synthesis have been identified (Kurdyukov et al., 2006; Javelle et al., 2011; Oshima et al., 2013). More indirect effects may also arise from perturbations in fruit cell division and development which affect cuticle formation in tomato fruit (Czerednik et al., 2012).

In addition to fruit brightness, the tomato cuticle mutants identified should be instrumental for deciphering the bases of structural, mechanical or water barrier properties of the cuticle (Schreiber, 2010; Dominguez et al., 2011) and their influence on fruit performances such as resistance to cracking, drought and pathogens. For example, the water loss properties of tomato fruit cuticle are largely affected by strong cutin alterations, as in the *gds12-b* mutant, though the associated modifications in cuticle structure have not been studied in detail (Isaacson et al., 2009; Girard et al., 2012; Yeats et al., 2012 and Fig. 6). Likewise, the high proportion of cyclic triterpenoids found in the wax-rich *glossy* (P4E2) or *dull* (P6D6) mutants may modify the water loss properties of the fruit (Vogg et al., 2004; Buschhaus and Jetter, 2012).

**Cuticle composition analysis of EMS mutants as a tool for discovering genes and pathways**

Insights into cuticle composition may give some hints on the regulations or biochemical pathways affected in the various cuticle mutants. Before undertaking allelism tests by crossing the various mutants, as done for the wax *glaucous* mutants in *Sorghum* (Peters et al., 2009), we first analyzed mutants for their cutin and wax composition (Table 2 and 3). Indeed, several groups of mutants displayed remarkably similar cuticle characteristics.
suggesting the involvement of the mutated genes in related biochemical pathways (e.g. in the cutin-deficient mutants P15C12 and P23F12) or in their regulation (e.g. in the wax- and cutin-rich mutants P18H8 and P30A12). These groups may first help discovering new genes and functions related to poorly known aspects of cuticle formation. In addition, as discussed below for the gds1 lipase gene, these mutants may help uncovering new alleles of known candidate genes such as those involved in cutin formation or in the coordinated regulation of wax and cutin biosynthesis (Yeats and Rose, 2013). Finally, these groups may also unravel genes involved in suberin biosynthesis. In the P5E1 and P26E8 mutants, the accumulation in cutin fraction of dicarboxylic acids, which are considered as markers for suberin, and the associated visual changes (Supplemental Fig. S6) may be indicative of the constitutive up-regulation of suberin biosynthetic pathway in these mutants. Indeed, the C18:1 unsaturated dicarboxylic acid content of P5E1 and P26E8 tomato cuticle (~ 99 µg.cm²; Supplemental table S2) is close to that of potato tuber periderm (~ 80-100 µg.cm²; Serra et al., 2009). Since both species are closely related, these mutants may thus prove very interesting for investigating the relationships between cutin and suberin within the Solanaceae. While both partially share a common biosynthetic pathway, suberin normally accumulates in the fruit only after wounding and in other stressful conditions.

Next step is the identification of the mutations underlying the mutant cuticle traits. Identification of unknown mutations in tomato classically proceeds through map-based cloning by crossing the S. lycopersicum mutant with a wild-related species such as S. pimpinellifolium (Isaacson et al., 2009; Yeats et al., 2012). To reduce the inherent high genetic and phenotypic variability in the offspring of such cross, we used as parental line a dwarf mutant of the cultivated M82 genotype, taking advantage of the development of SNP markers polymorphic between M82 and Micro-Tom (Shirasawa et al., 2010). Combination of genetic mapping of the mutation and candidate gene approach resulted in the identification of a new hypomorphic or null allele of the SlGDSL2 gene responsible for a strong cutin-deficient mutation. Sequencing of the SlGDSL2 gene from the WT and P15C12 plants revealed an A to T mutation disrupting the 3’ splice site of intron 4, which leads to the accumulation of unspliced mRNA and to mRNA resulting from the utilization of a cryptic 3’ splicing site in exon 5 and, in both cases, to truncated SlGDSL2 proteins. Thus, evidences presented here indicate that SIGDSL2 mutant plants are not able to accumulate correctly spliced mRNA in fruit epidermis. In both scenarios (no-splicing or missplicing of intron 4), the presence of premature translation-termination codons (PTC) in the mRNAs would result in the generation of truncated GDSL-lipases lacking the C-terminal region. This region has an essential role in SIGDSL2 activity since it harbours two residues of the GDSL lipase catalytic triad: the aspartic acid (D-321) and the histidine (H-326) (Akoh et al., 2004). Truncated
proteins devoid of GDSL lipase enzymatic activity could therefore have dominant-negative effects on cutin formation e.g. by interfering with a cutin-assembly machinery involving SIGDSL2. Actually, the very low SIGDSL2 transcript level and the absence of immuno-detected SIGDSL2 protein in gdsl2-b fruit epidermis (Fig. 7F) strongly suggest that the nonsense-mediated mRNA decay, a quality control mechanism present in plants and other eukaryots that recognizes and degrades aberrant mRNA harbouring PTC (Stalder et al., 2008), prevents the production of truncated SIGDSL2 proteins which could possibly have detrimental effects on the plant.

Interestingly, one of the three fruit cutin-deficiency (cd1) mutants identified in the M82 tomato mutant collection (Menda et al., 2004; Isaacson et al., 2009) was recently identified as a SIGDSL2 truncation mutant (Yeats et al., 2012). The discovery of SIGDSL2 mutations by two independent screening of unrelated tomato mutant collections in M82 and Micro-Tom is probably not a mere coincidence. SIGDSL2 was a likely candidate for cutin assembly in the cuticle due to its properties and its high and specific expression in tomato fruit epidermis (Lemaire-Chamley et al., 2005; Reina et al., 2007; Mintz-Oron et al., 2008; Matas et al., 2011). Reverse genetics approaches based on both screening of mutant collections in processing tomato and RNAi-silencing strategy in cherry tomato (Girard et al., 2012; Yeats et al., 2012) recently proved that SIGDSL2 plays a key role as acyl-transferase for the assembly of monomers to form the cutin polyester in the cuticle (Yeats and Rose, 2013). The new gdsll2 allelic variant described in this study will help investigating the role of the GDSL lipase in planta e.g. by allowing comparison of GDSL function in cutin polymerization in different genetic backgrounds (cultivated tomato S. lycopersicum, M82 and Micro-Tom; cherry tomato S. lycopersicum var. cerasiformae Wva 106).

In conclusion, tomato mutants characterized herein or present in tomato EMS mutant collections constitute rich sources of genetic variability for exploring cuticle properties and for identifying new genes and alleles involved in cuticle formation. As an example, several other strong cutin deficiency mutants identified in this study do not map with other known candidate genes. The recessive P23F12 mutation maps to chromosome 9 unlike other mutations identified to date in tomato (Supplemental Fig. S9). The P30B6 mutation, which maps in a different location to chromosome 9, displays a different inheritance pattern (overdominance, data not shown). These results open the way for the discovery of new genes involved in cutin formation in tomato. The continual technological and scientific advances in tomato genomics and in cuticle research now greatly facilitate the identification of the mutations underlying the mutant traits. In addition to traditional map-based cloning (Isaacson et al., 2009), combination of genetic mapping and candidate gene approach, which already proved successful (this study and Shi et al., 2013), can be further extended to the
identification of the most promising target genes by RNA seq analysis of cuticle fruit mutants. Another very promising approach is the identification of the mutation by whole genome sequencing (Abe et al., 2012; Just et al., 2013), which only involves crosses between WT Micro-Tom and mutant Micro-Tom and therefore excludes all other undesirable variations of other cuticle compounds that could alter cuticle properties.

MATERIALS AND METHODS

Plant materials

Fruit brightness mutants were isolated from an EMS (ethyl methanesulfonate) mutant tomato (Solanum lycopersicum) collection generated in the miniature Microtom cultivar at INRA Bordeaux (France) as previously described (Rothan and Causse 2007; Just et al., 2013). Plants were grown at a density of 200 plants per square meter in a greenhouse under 14-h-light/10-h-dark, 18°C to 28°C temperature and 60% to 80% relative humidity. Plants were watered twice a week with solution 1 [pH 5.8, oligoelements plus 3.5 mM KNO₃, 1 mM K₂SO₄, 2 mM KH₂PO₄, 6 mM Ca(NO₃)₂, 2 mM MgSO₄] until first fruit set on the first truss, then with solution 2 [pH 5.8, oligoelements plus 4 mM KNO₃, 1.5 mM K₂SO₄, 1.5 mM KH₂PO₄, 4 mM Ca(NO₃)₂, 1.5 mM MgSO₄] until fruits were ripe. Flowers were regularly vibrated to ensure optimal self-pollination and therefore fruit development. Seeds were treated with 4% calcium hypochlorite for 15 min, rinsed for 30 min, air-dried for 2 days and stored at 4°C with a desiccant until use.

Genetic mapping

A mapping F₂ population of 110 plants was created by crossing the P15C12 Micro-Tom glossy mutant with a dwarf mutant from the M82 cultivar EMS population (Menda et al., 2004). Plants from the mapping population were grown in greenhouse in the conditions indicated above, except that plant density was 21 plants per square meter and that plants were watered 3 times a week. Cuticle-associated traits were measured at two developmental stages of the fruit, first at the Mature Green stage (brightness and permeability) then at the Red Ripe stage (brightness, water loss, width of the cutin layer surrounding fruit epidermal cells). Genomic DNA was extracted from young leaves of each plant by a CTAB method. Five hundred µl of Cetyltrimethylammonium Bromide (CTAB) extraction buffer [Sorbitol 145 mM, 850 mM NaCl, 125 mM Tris-HCl (pH 8.0), 25 mM EDTA, Sarcosyl (n-lauroyl-sarcosine) 0.8% (w/v), CTAB 0.8% (w/v),] was added with 2.2 mg of sodium metabisulfite per sample, incubated at 65°C for 90 min, 450 µl of chloroform:isoamyl alcohol (24:1) was added to each
sample, mixed by inversion and centrifuged 10 min at 4500 g. Subsequently, the supernatant was transferred to a fresh tube and treated with 500µl of cold isopropanol. DNA was dried 15 min at 45°C under vacuum, suspended in 50µl of distilled water and samples were RNase-treated. Primer sets of 48 SNP markers well spread along the genome and displaying polymorphism between the Micro-Tom and the M82 cultivars (Shirasawa et al., 2010) were chosen for genotyping each plant. Genotype and phenotype data were analyzed using MapMaker /Exp v3.0 (Lander et al., 1987) and QTL Cartographer (Basten et al., 2002).

**Fruit brightness assessment**

Fruit brightness of the mutant lines was visually estimated at the Mature Green (MG) and Red Ripe (RR; Breaker stage plus 7 days) stages by comparison with wild type (WT) Micro-Tom plants grown side-by-side and disseminated randomly among mutant lines. Mutants differing from WT MicroTom were classified as glossy mutants, i.e. fruits with homogeneous glossy aspect, or as dull mutants, i.e. those with no light reflect on the fruit. An important criterion for classifying mutants as fruit brightness mutants was that all fruits on the plant displayed the same phenotype. However, several selected mutant lines carried both glossy and dull fruits, which were clearly different from the WT fruits. Analysis of fruit brightness in the segregating P15C12 Micro-Tom mutant X M82 dwarf population was done similarly except that fruit brightness was estimated by comparison of all genotypes to each other and that genotypes were further classified in three brightness groups.

**Water loss and cuticle permeability measurements**

For water loss measurements, one RR fruit was harvested from each plant of the F2 mapping population, including the parents. Fruit sealing wax was then applied on stalks and fruits were stored at room temperature. Fruit fresh weight was recorded at T0 and each week, until 6 weeks. Water loss was calculated as a percentage of weight loss. For measurements of cuticle permeability to stain, one MG fruit was collected from each plant of the F2 mapping population and from the parents, and dropped in 1% Toluidine Blue solution during 6-h as described in Tanaka et al., (2004).

**Cutin monomer and wax analysis**

Cuticular waxes were extracted by fruit immersion during 30 sec in 6 mL of chloroform containing 6 µg of docosane, as internal standard. Extracts were dried under moderate nitrogen flux and lipids were derivatized 15 min at 110°C in 100 µL of [N,O-bis (trimethylsilyl) trifluoroacetamide] : trimethylchlorosilane (99:1)] (BSTFA-TMCS). The BSTFA-TMCS was dried under nitrogen and samples were suspended in 500 µL of hexane. Wax was then analyzed and quantified as already described (Bourdenx et al., 2011).
For the cutin monomer analysis, a 1 cm diameter disk was cut off from a RR fruit epidermis, carefully scratched with a scalpel blade in order to remove exocarp cells and incubated 30 min in isopropanol at 85°C. The disk was then delipidated by successive deeps in chloroform / methanol (C/M), at different ratios: C/M (2:1) for 24h, C/M (1:1) for 24h, C/M (1:2) for 24h and 100% methanol for 24h. The delipidated epidermis disk was dried 48h under pulsed air and 48h in a desiccator. Cutin was then depolymerized, analyzed and quantified as already described (Domergue et al., 2010).

**Light microscopy**

For cuticle thickness measurements, fruit exocarp (including cuticle) was obtained from 2 independent RR fruits of WT and mutant lines. Samples were fixed and embedded in paraffin as previously described (Mounet et al., 2009). Eight micrometer slides of exocarp were stained using saturated and filtered Sudan Red solution in ethanol. Mean cutin thickness was assessed from 60 measurements.

For analyzing the thickness of the cutin layer between two adjacent fruit epidermal cells (thereafter called cutin width) in the mapping population, a 10 mm² square tissue sample was peeled off from one RR fruit harvested from each plant of the F₂ mapping population, including the parents. As much epidermal cells as possible were then removed from the peel by scratching the internal surface with a scalpel blade. The resulting cuticle-enriched fragment was immerged in water, placed flatten on a glass slide and observed under optical microscope at x20 magnification. Cutin width was then determined by measuring thickness of the cutinized cell wall between two adjacent epidermal cells (Supplemental Fig. 6). Mean cutin width was assessed from 12 measurements.

**Environmental Scanning Electron Microscopy (ESEM)**

For the observation of wax on the surface of the fruit, 5 mm side cubes of exocarp including cuticle were excised from the equatorial part of RR fruits and placed directly into the observation chamber of a scanning electronic microscope FEI Quanta 200, in environmental mode. For cutin observation, cubes were beforehand submerged 30 s into chloroform, with gentle agitation. Observations were performed with a plate at 4°C, a 6 Tor pressure in the chamber and a 4.7 V voltage applied to the filament. Pictures were captured with x600 and x3000 magnifications.

**PCR and Quantitative Reverse Transcription PCR analysis of SlGDSL2**

Genomic DNA was extracted from 100 mg fresh weight (FW) of WT and P15C12 mutant leaves with the Plant DNAzol reagent (Invitrogen), further amplified using 4 primer pairs, GDSL 1F/1R, GDSL 2F/2R, GDSL 3F/3R, GDSL 4F/4R, and sequenced for determination of
allelic sequences of \textit{SIGDSL2} and \textit{gds12-b}. Primer sequences are indicated in Supplemental Fig. S10.

The cDNA sizing was performed after a total RNA extraction from 100 mg FW and a reverse transcription, as described previously (Mounet \textit{et al.}, 2009; Girard \textit{et al.}, 2012). The amplification was performed using GDSL2-cDNA4F and GDSL2-cDNA5R primers. Primers sequences are indicated in Supplemental Fig. S10.

\textit{SIGDSL2} expression was analyzed from bulks of plants segregating for both the fruit brightness and the \textit{gds12-b} mutation in the F$_2$ mapping population. The WT Micro-Tom, the M82dwarf and the P15C12 (\textit{gds12-b}) parental lines were included in the analysis. The glossy fruit bulk (homozygous for the mutant \textit{gds12-b} allele) was constituted by 21 RR fruits collected from 7 plants (3 fruits per plant). On each fruit, two disks of 1 cm diameter were collected and carefully scratched with a scalpel blade. Three pooled samples were then generated by distributing two discs per plant in each pool \textit{i.e.} 14 discs per pooled sample in total. Same strategy was used for the WT-like bulk (\textit{SIGDSL2} allele) except that the three pooled samples were prepared from 24 RR fruits collected on 4 plants \textit{i.e.} 16 discs per pooled sample in total. Samples were ground in liquid nitrogen and stored at -80°C until RNA extraction as described previously (Mounet \textit{et al.}, 2009). PCR analysis was performed on reverse transcribed cDNAs as previously described (Girard \textit{et al.}, 2012), using primers GDSL2-53 and GDSL2-34. Primer sequences are indicated in Supplemental Fig. S10.

\textbf{SIGDSL2 immunoblot analysis}

Four independent 15 DPA fruit pools were constituted for both WT and \textit{gds12-b} mutant line P15C12. Each pool was composed of the outer epidermis from 3 fruits, ground and lyophilized. Antibody and immunoblot analyses were as previously described (Girard \textit{et al.}, 2012).

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Tables

**Table I.** Wax composition of fruit cuticle from wild type and selected tomato mutants. Mean values (µg.cm\(^{-2}\) x10) of total wax load and of individual compounds are given with SD (n=4). % of total wax load is indicated for individual compounds. Unidentified compounds are not included in the table.

| Plant | Totalload | Mean SD | n-Rhenes | Mean SD | Monothanines | Mean SD | Alcohol | Mean SD | Amyrins | Mean SD | FattyAcids | Mean SD | % |
|-------|-----------|---------|-----------|---------|--------------|---------|---------|---------|---------|---------|------------|---------|---|
| WT    | 60.7 ± 7.5 | 42.0 ± 4.7 | 60.0 | 4.7 ± 3.0 | 7.6 | 2.1 ± 0.5 | 3.4 | 1.10 ± 2.4 | 17.8 | 2.0 ± 0.5 | 3.3 |
| F2OC10 | 63.8 ± 3.6 | 41.4 ± 1.0 | 65.0 | 3.4 ± 3.1 | 5.4 | 1.5 ± 0.3 | 2.0 | 15.4 ± 3.5 | 24.2 | 2.0 ± 0.3 | 3.2 |
| F2OC6 | 69.8 ± 5.9 | 45.1 ± 4.0 | 64.0 | 5.4 ± 1.1 | 7.8 | 2.5 ± 0.2 | 3.6 | 13.3 ± 0.9 | 19.0 | 3.6 ± 1.1 | 5.0 |
| F2A12 | 74.1 ± 10.9 | 42.0 ± 4.5 | 60.0 | 5.3 ± 0.7 | 7.0 | 2.6 ± 0.2 | 3.9 | 18.6 ± 4.1 | 25.1 | 2.6 ± 0.5 | 5.8 |
| F5C1 | 75.0 ± 4.7 | 52.9 ± 3.2 | 70.0 | 5.4 ± 3.0 | 7.2 | 2.5 ± 0.2 | 3.0 | 15.5 ± 0.9 | 13.9 | 4.1 ± 0.6 | 5.4 |
| F4B1 | 76.0 ± 6.3 | 49.5 ± 3.8 | 65.4 | 5.1 ± 1.0 | 6.7 | 2.5 ± 0.7 | 3.0 | 15.3 ± 0.6 | 20.1 | 3.4 ± 1.2 | 4.5 |
| F5B1 | 78.8 ± 8.4 | 51.7 ± 5.5 | 65.6 | 4.3 ± 3.2 | 5.5 | 2.5 ± 0.6 | 3.2 | 18.1 ± 4.0 | 23.0 | 2.2 ± 0.2 | 2.7 |
| R2A2 | 78.9 ± 4.2 | 47.6 ± 3.0 | 60.1 | 6.8 ± 3.2 | 8.6 | 3.6 ± 0.7 | 4.6 | 15.9 ± 4.7 | 23.9 | 2.2 ± 0.5 | 2.8 |
| F5H1 | 83.8 ± 8.1 | 54.0 ± 6.0 | 64.4 | 5.6 ± 0.4 | 6.1 | 3.3 ± 1.0 | 3.8 | 19.0 ± 1.7 | 22.6 | 2.0 ± 0.7 | 2.4 |
| P4E1 | 86.5 ± 17.1 | 58.7 ± 11.6 | 62.0 | 5.1 ± 1.0 | 5.7 | 10.0 ± 3.5 | 11.6 | 13.1 ± 2.6 | 14.6 | 2.1 ± 0.5 | 2.9 |
| P1/C12 | 88.6 ± 12.9 | 53.8 ± 9.6 | 60.1 | 5.8 ± 1.0 | 6.5 | 3.0 ± 1.1 | 3.4 | 23.9 ± 2.5 | 28.9 | 1.1 ± 0.4 | 1.2 |
| P2OC12 | 92.5 ± 20.5 | 61.0 ± 9.6 | 66.0 | 5.3 ± 1.0 | 5.4 | 3.8 ± 1.1 | 4.1 | 20.1 ± 9.0 | 21.7 | 2.7 ± 0.4 | 2.9 |
| P1/O16 | 27.6 ± 12.7 | 17.2 ± 8.4 | 68.8 | 4.8 ± 2.4 | 4.9 | 3.2 ± 0.2 | 3.0 | 20.4 ± 4.7 | 20.9 | 2.0 ± 0.2 | 1.9 |
| F17/12 | 105.4 ± 14.9 | 62.2 ± 3.9 | 59.0 | 5.4 ± 3.5 | 5.1 | 4.4 ± 0.8 | 4.2 | 31.0 ± 15.1 | 29.4 | 2.5 ± 0.9 | 2.3 |
| P2O/C2 | 106.0 ± 19.8 | 51.7 ± 11.0 | 58.2 | 5.7 ± 0.6 | 5.3 | 5.6 ± 0.6 | 2.4 | 34.6 ± 7.1 | 32.6 | 1.6 ± 0.4 | 1.6 |
| F1H12 | 125.5 ± 32.6 | 64.0 ± 25.0 | 64.9 | 9.1 ± 1.4 | 7.0 | 9.0 ± 3.6 | 7.2 | 25.7 ± 5.4 | 17.5 | 4.4 ± 1.4 | 3.4 |
| F2O/C3 | 134.5 ± 7.0 | 71.4 ± 5.0 | 66.0 | 5.4 ± 1.4 | 4.9 | 7.4 ± 2.0 | 5.0 | 21.0 ± 2.9 | 20.1 | 3.2 ± 0.0 | 2.0 |

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**Table II.** Cutin monomer composition of fruit cuticle from wild type and selected tomato mutants. Mean values (µg.cm$^{-2}$) of total cutin load and of individual compounds are given with SD (n=4). ND: not detected. Unidentified compounds are not included in the table. % of total cutin load is indicated for individual compounds. Unidentified compounds are not included in the table.

| Plant | Total Load | Fatty Acids | % Dehydroacids | % α-hydroxy Acids | % Ceramides | % Phosphatidic Acids | % 2-hydroxy Fatty Acids | % Fatty Alcohols |
|-------|------------|-------------|----------------|------------------|-------------|---------------------|------------------------|-----------------|
| WT    | 586.1 ± 167.4 | 352.0 ± 5.6  | 6.0            | 31.0 ± 8.6       | 5.4          | 152.8 ± 26.2        | 11.2                    | 17.9 ± 3.8       |
| P8612 | 156.1 ± 16.4 | 118.3 ± 1.9  | 7.2            | 7.2 ± 1.1        | 4.4          | 91.1 ± 0.9          | 11.7                    | 6.3 ± 0.9        |
| P2506 | 266.0 ± 24.0 | 17.8 ± 2.1   | 6.1            | 19.6 ± 3.5       | 3.7          | 19.8 ± 2.0          | 7.0                     | 8.8 ± 0.5        |
| P2506C | 368.0 ± 50.0 | 13.3 ± 2.5   | 4.1            | 19.9 ± 5.2       | 5.0          | 28.9 ± 6.3          | 5.0                     | 8.8 ± 0.7        |
| P6412 | 380.0 ± 103.5 | 19.2 ± 4.6   | 4.8            | 24.2 ± 5.5       | 7.0          | 29.0 ± 9.4          | 7.0                     | 10.5 ± 1.3       |
| P9261 | 535.4 ± 51.1 | 18.6 ± 3.8   | 7.2            | 19.6 ± 4.7       | 4.8          | 353.7 ± 27.2        | 57.6                    | 7.6 ± 0.7        |
| P9262 | 602.6 ± 42.6 | 40.1 ± 5.4   | 6.7            | 26.5 ± 3.7       | 4.7          | 50.1 ± 3.1          | 6.3                     | 16.8 ± 1.1       |
| P525H | 625.6 ± 14.0 | 23.9 ± 5.0   | 3.7            | 36.6 ± 2.7       | 5.9          | 46.8 ± 10.3         | 18.8                    | 28.9 ± 6.0       |
| P616 | 701.0 ± 76.0 | 57.0 ± 12.2  | 8.2            | 30.9 ± 13.1      | 5.1          | 54.2 ± 3.3          | 7.7                     | 15.2 ± 3.8       |
| P3546 | 722.6 ± 122.9 | 37.5 ± 7.5   | 4.0            | 155.7 ± 10.3     | 9.1          | 40.7 ± 4.0          | 9.1                     | 24.9 ± 0.2       |
| P3541 | 776.4 ± 118.9 | 36.9 ± 9.0   | 5.0            | 132.2 ± 27.6     | 8.2          | 50.3 ± 10.1         | 8.0                     | 21.1 ± 1.9       |
| P1102 | 778.5 ± 56.9 | 30.0 ± 19.8  | 3.9            | 26.4 ± 4.8       | 3.7          | 143.7 ± 17.1        | 18.1                    | 21.7 ± 3.5       |
| P2063 | 883.4 ± 122.9 | 36.5 ± 5.0   | 4.0            | 139.8 ± 61.1     | 15.7         | 77.5 ± 5.3          | 8.6                     | 30.7 ± 4.1       |
| P35412 | 956.4 ± 180.0 | 62.3 ± 14.0  | 8.6            | 41.8 ± 14.7      | 4.4          | 142.9 ± 34.1        | 14.9                    | 34.8 ± 1.8       |
| P51712 | 985.9 ± 134.7 | 56.3 ± 23.5  | 6.7            | 39.5 ± 9.0       | 5.0          | 112.8 ± 17.9        | 11.4                    | 45.9 ± 0.6       |
| P51712 | 1175.2 ± 236.6 | 79.5 ± 10.0  | 3.4            | 192.4 ± 14.0     | 9.0          | 81.7 ± 18.0         | 8.5                     | 36.9 ± 2.2       |

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**Table III.** Summary of the major locus identified for fruit brightness mutant P15C12 on chromosome 11 between markers 11289_715 and 10722_814. LOD Score=maximum logarithm of Odds for the presence of a locus controlling each analyzed trait by Simple Interval Mapping (SIM, MapmakerQTL). $R^2$ is the percentage of variance explained by the locus. $a$ is the additive allele effect.

| Trait            | LOD score | $R^2$ (%) | $a$    |
|------------------|-----------|-----------|--------|
| Permeability     | 15.90     | 75.3      | 1.2807 |
| Brightness       | 13.64     | 61.1      | 0.6287 |
| Width cutin      | 4.76      | 24        | -3.715 |
| Waterloss        | 8.13      | 65.6      | -25.885|

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Figure legends

Figure 1. Strategy used for the selection of the fruit brightness tomato mutants through the Microtom Mutant Data Base (MMDB) tool. Visual selection of fruit brightness mutants was done in comparison with wild type fruits at Red Ripe stage.

Figure 2. Total wax load in fruit cuticle from wild type and selected tomato mutants. Mean values (µg.cm²) are given with SD (n=4). Unidentified compounds are not included. Black bar: wild type; light grey bar: *glossy* mutants; dark grey bar: *dull* mutants. * indicates significant difference with wild type fruit (t test, P<0.01).

Figure 3. Total cutin load in fruit cuticle from wild type and selected tomato mutants. Mean values (µg.cm²) are given with SD (n=4). Unidentified compounds are not included. Black bar: wild type; light grey bar: *glossy* mutants; dark grey bar: *dull* mutants. * indicates significant difference with wild type fruit (t test, P<0.01).

Figure 4. Hierarchical Clustering Analysis (HCA) plot of the two first principal components showing the 24 mutants and wild type (WT) analyzed for cutin composition of fruit cuticle. Data were centered and scaled for the Principal Component Analysis (PCA). Euclidian distance and Ward aggregation method were used for the HCA. X and Y axes are the two principal components with the percentage of contribution to the two dimensions indicated between brackets. Cluster 1 is represented with black circles, cluster 2 with white triangles, cluster 3 with black crosses, cluster 4 with white diamonds, cluster 5 with white squares and cluster 6 with white circles. In bold: wild type and fruit brightness mutants further analyzed for fruit surface and cuticle.

Figure 5. The surface and cuticle from wild type (WT) and selected *glossy* and *dull* mutants fruits at Red Ripe stage. A, Light macroscopy images showing cuticle brightness; scale bar = 5 mm. B, Environmental scanning electron microscopy of native fruit surface; scale bar = 40 µm. C, Environmental scanning electron microscopy of de-waxed fruit surface; scale bar = 40 µm. D, Light microscopy of fruit exocarp sections stained with Sudan IV; scale bar = 30 µm. Black arrows show examples of cuticle thickness measurement points. E, Cuticle thickness measurements of pericarp sections stained with Sudan IV. Mean values (µm) of 40 measures on 3 different sections are given with SD.

Figure 6. Cuticle properties of the *glossy* P15C12 cutin-deficient mutant. A, Total cutin load of wild type (WT) and P15C12 fruits at 17 DPA (days post anthesis), 25 DPA, Mature Green (MG), Breaker (Br) and Red Ripe (RR) stages. Mean values (µg.cm²) of total cutin load are given with SD (n=4). B, Water loss of RR fruit during storage. Fruits were stored at 28°C under pulsed air, and masses were measured at T₀, and after 4, 7, 12, 15, 27 and 34 days of
storage. C, Permeability to toluidine blue of MG fruits incubated with the dye during 6h. D, Cutin width measurements on fresh pealed outer epidermis from RR fruits are expressed in μm; scale bar = 30μm. Twelve independent measures of the width of the cutinized cell walls were done after picture acquisition.

**Figure 7.** Mapping of the P15C12 glossy mutant and characterization of the mutation. A, the four cuticle-related traits (brightness, water loss, permeability, cutin width) were mapped to chromosome 11 between SNP markers 11289_715 and 10722_814. Genetic distance (cM) and physical position (ITAG2.40) are indicated on the left. Markers are indicated on the right. 1-LOD and 2-LOD support intervals of each QTL are marked by boxes and thin bars, respectively. B, SIGDSL2 gene splicing model. Exons, introns and splicing sites (in bold) are represented by white boxes, grey lines and white triangles respectively. The black triangle shows the A=>T mutation in the 3’ consensus splice site of intron 4, which results in a broken splicing site in the gds12-b mutant. The 3’ cryptic splicing site is shown on the right. C, Amino acid sequences deduced from native SIGDSL2 transcript (wild type plant, SIGDSL2 gene), unspliced transcript (in-frame reading through intron 4 in the gds12-b mutant) and alternative cryptic site transcript (splicing of intron 4 at 3’ cryptic site in exon 5 in the gds12-b mutant). Incorrect aminoacids are indicated in bold. D, Electrophoretic analysis of the SIGDSL2 transcripts in M82dwarf (M82d), wild type Micro-Tom (WT MT), gds12-b mutant and F1 hybrid between M82d and gds12-b. E, real-time RT-PCR analysis of SIGDSL2 expression in 20 DPA fruits in wild type Micro-Tom (WT, grey box) and gds12-b mutant (white box). AU: arbitrary units. Mean values of three biological replicates are shown with SD. * indicates significant difference with WT fruits (t test, P<0.01). F, Immunoblot analysis of GDSL2 protein in 4 independent pools of gds12-b and WT epidermis. Positive control is a recombinant GDSL extract (Girard et al., 2012).

**Figure 8.** Model of the influence of cuticle architecture on tomato fruit brightness. A, Light microscopy of fruit exocarp sections stained with Toluidin blue. B, Schematic representation of the architecture of fruit epidermis in wild type (WT), high and low cutin load glossy mutants and dull mutants. Epidermal cell (EC) are encased in cutinized cell walls (Cut, in dark grey) covered by waxes (W, light grey). C, Light reflection on tomato fruit surface. Black lines: incident light and specular reflection. Light grey lines: diffuse reflection.
**Supplemental data**

Supplementary data can be found at *Plant Physiol* online.

**Supplemental Figure S1.** Comparison of fruit brightness from selected glossy and dull mutants at Red Ripe stage. Wild type reference fruit is shown on the left; glossy mutants are shown in the upper panel; dull mutants are shown in the lower panel. Photographs were taken in standardized conditions.

**Supplemental Figure S2.** Comparison of the fruit brightness of mutants that showed the most extreme wax and cutin compositions.

**Supplemental Figure S3.** Variables factor map for cutin components, after a centered PCA analysis.

**Supplemental Figure S4.** Individual factor map after Principal Component Analysis (PCA) of the wax and cutin compositions. X and Y axes are the two principal components with the percentage of contribution to the two dimensions indicated between brackets. The brightness and associated samples are indicated in bold and underlined for the reference, in black for dull brightness and in bold grey for glossy brightness. The data were centered and scaled for the PCA.

**Supplemental Figure S5.** Variables factor map for wax and cutin components, after a centered PCA analysis. Black arrows and text: variables for cutin data; grey arrows and text: variables for wax data.

**Supplemental Figure S6.** Photograph of fruits from the P26E8 mutant showing suberin deposition at the distal end of the fruit.

**Supplemental Figure S7.** Microscopic observation of fresh peeled outer epidermis from Red Ripe fruit. Twelve independent measures of the width of the cutinized cell walls were done as indicated by the red arrows. Scale bar = 10 µm.

**Supplemental Figure S8.** Phenotyping the F2 progeny from the cross M82d x P15C12 . A, Visual scoring of fruit brightness performed on Mature Green fruits. B, Distribution of the F2 progeny for fruit brightness. C, Visual scoring of cuticle permeability to toluidine blue. Intensity of the blue coloration of the fruit surface was visually assessed for scoring fruit permeability. D, Distribution of the F2 progeny for cuticle permeability. E, Water loss in fruit kept for 6 weeks in ambient air at room temperature. Fresh weight of the fruit was measured initially and after the 6 weeks storage period. F, Distribution of the F2 progeny for water loss. Results are expressed as percentage of the initial mass of the fruit. G, Photographs of
epidermal cells from fruits from the F2 progeny ranged in the different cutin width classes. Measurements were done as indicated in Supplemental Fig. S7 on fresh peeled outer epidermis of Red Ripe fruits. Twelve independents measures of the width of the cutinized cell wall were done (20x magnification). H, Distribution of the F2 progeny for cutin width. Red arrows indicate the values for M82d, P15C12 mutant and their F1 hybrid.

**Supplemental Figure S9.** Mapping of the P23F12 glossy mutant. The three cuticle-related traits (brightness, permeability, cutin width) were mapped to chromosome 9 as indicated in Materials and Methods for the P15C12 mutant. Genetic distance (cM) is indicated on the left. Markers are indicated on the right. 1-LOD and 2-LOD support intervals of each QTL are marked by boxes and thin bars, respectively.

**Supplemental Figure S10.** Nucleotide sequences of the primers used in this study. A, Table of the primer sequences. B, Representation of the SIGDSL2 genomic sequence with the position of the primers used for mutation detection. C, Representation of the SIGDSL2 coding sequence with the primers used for cDNA sequencing.

**Supplemental Table S1.** Comparison of the plant and fruit brightness data obtained from the MMDB database and from the subsequent observation of the 40 selected families grown in the greenhouse. Fruit brightness was visually assessed by comparison with wild type fruit at Mature Green and Red Ripe stages.

**Supplemental Table S2.** A, Cutin composition of fruit cuticle from wild type and 4 selected tomato mutants. Mean values (µg.cm⁻²) of each compound are given with SD (n=4). B, Wax composition of fruit cuticle from wild type and 4 selected tomato mutants. Mean values (µg.cm⁻² x10) of each compounds are given with SD (n=4). ND: not detected. Unidentified compounds are not included in the table.

**Supplemental Table S3.** Mean density of fruit epidermal cells in the indicated mutants. Cell counting was performed on 4 independent sections (0.09 mm²) of Red Ripe fruit pericarp observed under microscope at 10x magnification after staining with 0.1% toluidine blue. Mean values are given with SD (n=4).
Figure 1. Strategy used for the selection of the fruit brightness tomato mutants through the Microtom Mutant Data Base (MMDB) tool. Visual selection of fruit brightness mutants was done in comparison with wild type fruits at Red Ripe stage.
Figure 2. Total wax load in fruit cuticle from wild type and selected tomato mutants. Mean values (µg.cm⁻²) are given with SD (n=4). Unidentified compounds are not included. Black bar: wild type; light grey bar: glossy mutants; dark grey bar: dull mutants. * indicates significant difference with wild type fruit (t test, P<0.01).
Figure 3. Total cutin load in fruit cuticle from wild type and selected tomato mutants. Mean values (µg·cm⁻²) are given with SD (n=4). Unidentified compounds are not included. Black bar: wild type; light grey bar: glossy mutants; dark grey bar: dull mutants. * indicates significant difference with wild type fruit (t test, P<0.01).
**Figure 4.** Hierarchical Clustering Analysis (HCA) plot of the two first principal components showing the 24 mutants and wild type (WT) analyzed for cutin composition of fruit cuticle. Data were centered and scaled for the Principal Component Analysis (PCA). Euclidian distance and Ward aggregation method were used for the HCA. X and Y axes are the two principal components with the percentage of contribution to the two dimensions indicated between brackets. Cluster 1 is represented with black circles, cluster 2 with white triangles, cluster 3 with black crosses, cluster 4 with white diamonds, cluster 5 with white squares and cluster 6 with white circles. In bold: wild type and fruit brightness mutants further analyzed for fruit surface and cuticle.
Figure 5. The surface and cuticle from wild type (WT) and selected glossy and dull mutants fruits at Red Ripe stage. A, Light microscopy images showing cuticle brightness; scale bar = 5 mm. B, Environmental scanning electron microscopy of native fruit surface; scale bar = 40 µm. C, Environmental scanning electron microscopy of de-waxed fruit surface; scale bar = 40 µm. D, Light microscopy of fruit exocarp sections stained with Sudan IV; scale bar = 30 µm. Black arrows show examples of cuticle thickness measurement points. E, Cuticle thickness measurements of pericarp sections stained with Sudan IV. Mean values (µm) of 40 measures on 3 different sections are given with SD.
Figure 6. Cuticle properties of the *glossy* P15C12 cutin-deficient mutant. A, Total cutin load of wild type (WT) and P15C12 fruits at 17 DPA (days post anthesis), 25 DPA, Mature Green (MG), Breaker (Br) and Red Ripe (RR) stages. Mean values (µg.cm²) of total cutin load are given with SD (n=4). B, Water loss of RR fruit during storage. Fruits were stored at 28°C under pulsed air, and masses were measured at T₀, and after 4, 7, 12, 15, 27 and 34 days of storage. C, Permeability to toluidine blue of MG fruits incubated with the dye during 6h. D, Cutin width measurements on fresh peeled outer epidermis from RR fruits are expressed in µm; scale bar = 30µm. Twenty independent measures of the width of the cutinized cell walls were done after picture acquisition.
Figure 7. Mapping of the P15C12 glossy mutant and characterization of the mutation. A, the four cuticle-related traits (brightness, water loss, permeability, cutin width) were mapped to chromosome 11 between SNP markers 11289_715 and 10722_814. Genetic distance (cM) and physical position (ITAG2.40) are indicated on the left. Markers are indicated on the right. 1-LOD and 2-LOD support intervals of each QTL are marked by boxes and thin bars, respectively. B, S IGDSL2 gene splicing model. Exons, introns and splicing sites (in bold) are represented by white boxes, grey lines and white triangles respectively. The black triangle shows the A=>T mutation in the 3’ consensus splice site of intron 4, which results in a broken splicing site in the gds2-b mutant. The 3’ cryptic splicing site is shown on the right. C, Amino acid sequences deduced from native SIGDSL2 transcript (wild type plant, SIGDSL2 gene), unspliced transcript (in-frame reading through intron 4 in the gds2-b mutant) and alternative cryptic site transcript (splicing of intron 4 at 3’ cryptic site in exon 5 in the gds2-b mutant). Incorrect aminoacids are indicated in bold. D, Electrophoretic analysis of the SIGDSL2 transcripts in M82dwarf (M82d), wild type Micro-Tom (WT MT), gds2-b mutant and F1 hybrid between M82d and gds2-b. E, real-time RT-PCR analysis of SIGDSL2 expression in 20 DPA fruits in wild type Micro-Tom (WT, grey box) and gds2-b mutant (white box). AU: arbitrary units. Mean values of three biological replicates are shown with SD. * indicates significant difference with WT fruits (t test, P<0.01). F, Immunoblot analysis of GDSL2 protein in 4 independent pools of gds2-b and WT epidermis. Positive control is a recombinant GDSL extract (Girard et al., 2012).
Figure 8. Model of the influence of cuticle architecture on tomato fruit brightness. A, Light microscopy of fruit exocarp sections stained with Toluidin blue. B, Schematic representation of the architecture of fruit epidermis in wild type (WT), high and low cutin load *glossy* mutants and *dull* mutants. Epidermal cell (EC) are encased in cutinized cell walls (Cut, in dark grey) covered by waxes (W, light grey). C, Light reflection on tomato fruit surface. Black lines: incident light and specular reflection. Light grey lines: diffuse reflection.