STRUCTURAL POLYMERS AND MOLECULAR
PATHWAYS THAT INFLUENCE TOMATO FRUIT
INTEGRITY AND SURFACE QUALITY TRAITS

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Structural Polymers and Molecular Pathways That Influence Tomato Fruit Integrity and Surface Quality Traits

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Plant cuticles are protective, hydrophobic, waxy coverings produced by the epidermal cells of aerial organs of land plant. The variation in fruit cuticle structure or composition may influence many commercially important fruit traits, such as storability/shelf life, surface glossiness, and resistance to desiccation, microbial infection or cracking. Despite the fundamental importance of the fruit cuticle, current knowledge of its synthesis and metabolism is relatively limited. This work describes the cloning and characterization of four tomato fruit cuticle-related mutants, including three cutin-deficient mutants (cd1, cd2 and cd3) and one early fruit cracking mutant (hcr1), each of which provides new insights into fruit cuticles and associated structural polymers.

In the first part of this thesis, the different aspects of plant cuticle biology, such as cuticle structure, functions, monomer biosynthesis and assembly are briefly reviewed, summarizing present knowledge of the cutin biosynthesis pathway. The second part of this research focuses on the map-based cloning of three cutin-deficient mutants. The cd1 mutant was identified
as having a lesion in a gene in the GDSL-motif lipase/hydrolase family. Further research has demonstrated that the CD1 enzyme catalyzes extracellular cutin polymerization at the cuticle formation site. The cd2 gene was identified as a putative transcription factor in the IV group of the homeodomain-leucine zipper family (HD-Zip IV). The cd3 gene was identified as a member of CYP86A group in cytochrome P450 family, with possible catalytic activity of ω-hydroxylation to C16 and C18 fatty acids in the cutin biosynthesis pathway. The last part of this research focuses on the detailed characterization of hypercracking 1 (hcr1) mutant, which exhibits early fruit cracking and subsequent massive suberin deposition on the fruit surface. The HCR1 gene encodes a 3β-hydroxysteroid dehydrogenase/C-4 decarboxylase (3β-HSD/D1), which is a key enzyme in the sterol biosynthesis pathway. Sterol profiling showed that the mutation results in 11~16% reduction of sterol compositions in hcr1 mutant fruits. Phenotypic and biochemical analysis of the hcr1 mutant suggests that early fruit cracking probably results from the severe inhibition of cell division/expansion in pericarp. The possible physiological and molecular mechanisms underlying the abnormal cell expansion/division in hcr1 fruit are discussed.
BIOGRAPHICAL SKETCH

Yonghua He was born September 23rd, 1964 in Sichuan Province, People’s Republic of China. He earned his B.S. in Botany in 1985 from the Department of Biology, Lanzhou University, Lanzhou China. In the same year, he started his career as a botanist at the Chengdu Institute of Biology (CIB), Chinese Academy of Sciences (CAS). During his tenure at the CIB, He was engaged in studies of the systematics and natural resources of Genus Vitis and Rosa, and studies on restoration and reconstruction of degraded mountain ecosystems. He came to Cornell in 2002, and had worked in Dr. Timothy Fahey’s Lab in the Department of Natural Resources for three years. His work involved in some ecological projects related to rhizosphere carbon and nitrogen dynamics in forest ecosystems. In 2006, he joined Dr. Rose’s research team in the Department of Plant Biology at Cornell. Since then he has been engaged in genetic and molecular biology studies of tomato. In the spring of 2008, he entered the Ph.D. program in the Department of Plant Biology at Cornell University, where he received his doctorate in the Horticulture field in December, 2011.
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CHAPTER ONE: LITERATURE REVIEW

Plant Surface Lipid Barrier: Function, Structure, Synthesis and Assembly

The plant cuticle, defined as an extracellular lipid-rich membrane covering aerial organs of plants, is a crucial adaptation of terrestrial plants to their harsh environment on land (Riederer, 2006). The cuticle can also be considered as a cutinized cell wall, because of the nature of its cutin-rich composition and intensive interaction with the underlying polysaccharide cell wall (Dominguez, 2011). The plant cuticle serves mainly as a physical barrier to minimize water loss and pathogen infection, and also to protect the plant against other physical, chemical and biological threats. However, despite its biological importance, some aspects of cuticle biology remain obscure. The studies of plant cuticle biology have been recently reviewed and discussed in detail in several papers (Pollard et al., 2008; Kerstin Koch et al., 2008; Buschhaus and Jetter, 2011; Dominguez, 2011; Javelle, 2011). The different aspects of plant cuticle biology, such as structure, potential functions, monomer biosynthesis and assembly will be summarized in this review.
1.1 The Structure and Composition of Plant Cuticles

1.1.1 The structure of plant cuticle

The plant cuticle is a hydrophobic lipid layer secreted by epidermal cells on their outer surfaces (Fig. 1.1). The biochemical composition of plant cuticles varies among species or organs, but they are basically composed of two main components: cutin and waxes (Jeffree, 2006; Nawrath, 2006). Moreover, the structural organization of the components also follows a similar pattern: a thin epicuticular wax layer on the outer surface, beneath which is a cutin matrix, infiltrated with intracuticular waxes, phenolics and polysaccharides (Jeffree, 2006; Dominguez, et al., 2010) (Fig. 1.1D). Epicuticular waxes are deposited as a relatively uniform and amorphous layer, or in crystal form. The matrix underneath is mainly composed of cutin (accounting for 40–80% by weight). The thickness of this cutin matrix can range from less than 1 µm to 10 µm or more, depending on the species. Intracuticular waxes and phenolic compounds are embedded or trapped in the cutin matrix. On the inner side of the cuticle, cutin is mixed with polysaccharides, such as cellulose, hemicellulose and pectin, which are secreted into the epidermal cell wall. The nature of the interconnections between cutin and cuticle polysaccharides, including specific intermolecular, is currently unclear.
Figure 1.1  The structure of plant cuticle.  (A) Light micrograph of the cuticle of tomato fruit (cv. M82) stained with Sudan IV. Scale bar = 20 μm.  (B) A 3-D rendering of the tomato fruit cuticle. Scale bar = 10 μm.  (C) Transmission electron micrograph of the tomato fruit (cv. M82) cuticle. Scale bar = 500 nm.  (D) Structural model of a transverse section of plant cuticle, showing the different components (adapted from Dominguez et al., 2011).
1.1.2 The major compositions of plant cuticle

Cutin, a biological polymer which is cross-linked mainly by ester bonds between its monomers, serves as the structural backbone of the plant cuticle (Nawrath, 2006; Dominguez, et al., 2011) (Fig. 1.2B). Overall, the dominant aliphatic monomers of plant cutin are C16 and C18 ω-hydroxylated fatty acids or dicarboxylic acids (DCA), with or without mid-chain oxygenated substitutions, such as hydroxyl or epoxy groups (Fig. 1.2A). In most plants, the C16 cutin monomers are typically composed of 16-hydroxy-hexadecanoic acid and 9(10), 16- dihydroxyhexadecanoic acid. The main components of the C18 cutin monomers are 9, 10, 18-trihydroxyoctadecanoic acid and 18-hydroxy-9, 10-epoxyoctadecanoic acid, together with their monounsaturated homologs (Fig. 1.2A) (Pollard et al., 2008; Dominguez, et al., 2010). In Arabidopsis thaliana, however, dicarboxylic acids (DCA) and glycerol are the predominant cutin monomers. Some very long chain ω-hydroxy fatty acids (C20–C28) have also been identified in the cutin of this model plant (Pollard et al., 2008).

Although the polymerization models of cutin monomers have been proposed (Kolattukudy, 1977; Ramirez et al., 1992), several important aspects of cutin polymer structure are still unclear (Pollard et al., 2008). For instance, it is unknown if cutin exists as discrete polymer molecules or as a highly cross-linked continuum. In some plants, the dominant cutin monomers are
Figure 1.2  Cutin monomers and proposed cutin networking.
(A) Major cutin monomers of C16 and C18 in the plant cuticle.
(B) Proposed schematic networking of a cutin polymer.
(Adapted from Dominguez et al., 2011)
ω-hydroxy fatty acids, where self-polymerization will produce a linear polyester chain. In the case of ω-hydroxy fatty acids with mid-chain oxygen-containing functional groups (epoxy, hydroxy or vicinal diol), the mid-chain hydroxyls may be esterified to create a branched structure on the linear polyester chain (Kolattukudy 2001) (Fig. 1.2B). In Arabidopsis, glycerol has been found in the cutin matrix, prompting speculation that the glycerol is probably directly esterified with DCAs or ω-hydroxy fatty acids to produce a crosslinked domain or dendrimer structure. However, this kind of polymerization of cutin monomers is considered as an atypical or non-representative pattern in the majority of plants (Graca et al., 2002; Franke et al., 2005; Pollard et al., 2008).

**Waxes** are collectively a structurally diverse mixture of discrete hydrophobic compounds that are primarily composed of aliphatic lipids and their derivatives (Nawrath, 2002; Kunst and Samuels, 2003; Kerstin Koch et al., 2008). Generally, both epicuticular and intracuticular waxes are a mixture of primary and secondary alcohols, \( n \)-aldehydes, \( n \)-alkanes, ketones, wax esters and very long chain fatty acids (VLCFAs) with chain lengths typically in the range C20 to C34, and in the case of wax esters (two connected chains), about 60 carbons. Several reviews have addressed the chemical composition of plant waxes in detail, and a long list of rare and uncommon wax compositions has been reported (Baker, 1982; Riederer and Markstadter, 1996; Kunst and Samuels, 2003; Jetter et al., 2006). In addition, other
compounds, such as triterpenoids and phenylpropanoids also can be detected in the wax fraction.

The epicuticular wax may be deposited as a film or in the form of crystals. In contrast, intracuticular waxes are consider to be embedded into cutin and can be extracted by organic solvents. Direct evidence has suggested that the compositional differences between epicuticular and intracuticular waxes are significant in both quality and quantity. The mechanism causing this wax fractionation difference remains unclear but it may be influenced, at least in part, by spontaneous partitioning due to the physicochemical properties of the wax compounds and interactions with other intracuticular polymers (Buschhaus and Jetter, 2011). In addition, it has been observed that the wax composition of the plant cuticle is highly variable among the organs or plant species, and may also be significantly altered by some environmental factors (Jetter and Schaffer, 2001; Cameron et al., 2006).

**Cutan** is non-saponifiable lipid polymer existing in the cuticle of some plants, either as a substitute for, or in combination with cutin (Kolattukudy, 1996). A common characteristic of cutan polymers is their resistance to de-esterification by alkaline hydrolysis (e.g. hot BF₃/methanol). Cutan is often considered to be composed of poly-unsaturated fatty acid derivatives, typically linked to each other by ether bonds, as distinct from the ester bonds that exist in cutin polymers (Villena et al., 1999).
Cutan is present in varying amounts in the cuticle of different plants or organs, and can account for at least as much mass as cutin in some species (Fernandez et al., 2011). Many cutan-rich plants, such as some crassulacean acid metabolism (CAM) plants, have a certain degree of adaptation to drought. This suggests that cutan may enhance the hydrophobic nature of the cuticle, thus more effectively preventing water loss from the plant organs (Boom et al., 2005). Little is known about cutan biosynthesis and assembly, and its functional roles to protect plants from harsh environmental conditions.

**Phenolics** only account for a small portion (typically a few percent) of the plant cuticle and are principally composed of cinnamic acids, flavonoids or lignin-like chemicals, and (Hunt and Baker, 1980; Reina et al., 2001). The phenolic compounds can absorb UV light, thus providing a protective function against UV radiation. Their distribution in the flower cuticle may increase pollinating insect attraction (Kolb and Pfundel, 2005; Koch et al., 2008).

### 1.2 The Function of the Plant Cuticle

The highly hydrophobic cuticle layer plays important roles in plant growth and development, essentially as a barrier to control the movement of water, gases and solutes, and to protect against pathogen infection. The plant cuticle is also involved in other functions, such as preventing organ fusion, protecting against mechanical injury and attenuating UV light absorption.
(Kerstiens, 1996; Riederer, 2006). Here, the major physiological functions of plant cuticles will be discussed.

1.2.1 An efficient diffusion barrier for water, gases and other molecules

The primary and most important function of the plant cuticle is considered to be as an efficient barrier for restricting the diffusion of water, gases or solutes across aerial plant organ surfaces (Schreiber, 2010). This idea is supported by the fact that mutants with severe defects in cuticle biosynthesis often do not survive under normal growth conditions, but may be rescued under conditions of high humidity (Tanaka et al., 2001; Yang et al., 2008).

The relative importance of cutin or waxes in restricting water loss is still debatable. Some Arabidopsis cutin-defect mutants, such as the gpat4/gpat8 double mutant, exhibit a high permeability to water but display a normal wax profile (Li et al., 2007). In contrast, the high epidermal permeability of the wax2 mutant cuticle is likely associated with a major reduction of its wax load (Chen et al., 2003; Rowland et al., 2007). As I report here, although three tomato cutin-deficient (cd) mutants all exhibited dramatic (95–98%) reductions in cutin content, transpirational water loss from the fruit at room temperature in high humidity conditions increased markedly only in cd1 (Fig. 1.3), but slightly in cd2 and cd3, compared with the wild type (Isaacson et al., 2009). This result suggests that there is probably no simple linear relationship between the
Figure 1.3 The function of plant cuticle: protecting against water loss. Differential water loss of tomato fruits between wild-type (M82) and cd1 mutant after storage for 21 days at room temperature (about 20°C). Scale bars = 1 cm.
amount of cutin and transpirational water loss in the fruit. Nevertheless, cuticular permeability is likely not only correlated to its physical thickness, chemical composition and structural assembly (Burghardt and Riederer, 2006), but also influenced by environmental factors, such as temperature and humidity (Shepherd and Griffiths, 2006).

1.2.2 A physical barrier to resist pathogen infection

The plant cuticle provides a physical barrier that resists penetration by virus particles, bacterial cells, and the spores or growing filaments of fungi. The function of the cuticle in resisting pathogen infection has been deduced from the following observations: some cuticular-defect mutants exhibit an increase in sensitivity to pathogens (Li et al., 2007; Lee et al., 2009), and some fungal pathogens secrete cutinases (a cutin hydrolases) to facilitate their penetration of the plant cuticle during colonization (Kolattukudy et al., 1995).

Interestingly, an Arabidopsis cutin defective mutant, sma4, displays an enhanced susceptibility to the avirulent strains of Pseudomonas syringae pv tomato, normal susceptibility to the biotrophic fungal pathogen Erysiphe cichoracearum, and an enhanced resistance to a necrotrophic fungal pathogen Botrytis cinerea (Tang et al., 2007). Similarly, other three Arabidopsis cutin-defective mutants, att1, bodyguard and lacerate, were also reported to show increased resistance to B. cinerea in the same experiment. This unexpected phenomenon might be explained by an enhanced perception
of putative elicitors leading to the production of antifungal compounds in those cutin-defective mutants, or by activating compensatory defense pathways, such as increased wax biosynthesis or the up-regulation of defense genes (Bessire et al., 2007; Chassot et al., 2007). In our studies, fruits of the tomato cutin-defective mutants (cd1, cd2 and cd3) displayed an enhanced susceptibility to B. cinerea under controlled inoculation conditions (Fig. 1.4), suggesting that the increased susceptibility of cd mutants probably resulted from a massive reduction in cutin load (Isaacson et al., 2009).

It is clear that the plant cuticle plays important roles in resistance against microbial infection. However, some aspects, such as the roles of cuticle composition and structure, and the interaction between pathogens and plants through cuticle, are still not well understood.

1.2.3 A physical barrier to prevent organs from fusion

The plant cuticle provides a highly hydrophobic barrier, which physically defines organ boundaries (Javelle et al., 2011) and without which organ fusion can occur. Organ fusion phenotypes have been observed in many cuticle-defective mutants, such as fdh, lcr, hth, wax2, ad1, wbc11, wsl1 and bgd (Lolle et al., 1998; Sinha and Lynch, 1998; Krolikowski et al., 2003; Bird et al., 2007; Yu et al., 2008). A typical organ fusion mutant, fdh, is characterized by the fusion of leaves, floral organs and ovules, even though the epidermal cell layer of these organs is intact (Lolle et al., 1992) (Fig. 1.5). Genetic studies showed
Figure 1.4 The function of plant cuticle: protecting against pathogen infection. The appearance of the ripe fruits of M82 and cd mutants after storage for 7 days following infection with *Botrytis cinerea.* (Pictures provided by Dr. Tal Isaacson. Scale bars = 1cm)
that the \textit{fdh} gene encodes a subunit of a fatty acid elongase complex (FAE), 3-ketoacyl CoA synthase (KCS), which is a key enzyme involved in cuticle monomer biosynthesis. Together, these results suggest that the organ fusion of \textit{fdh} results from defective cuticle formation (Fig. 1.5) (Pruiit et al., 2000). It should be mentioned that many cuticle-defective mutants do not exhibit an organ fusion phenotype. In addition, the respective roles of wax and cutin on organ separation and the precise mechanism underlying plant organ fusion are still obscure (Nawrath, 2002; Pighin et al., 2004).

\textbf{Figure 1.5 The function of plant cuticle: protecting against organ fusion.} Light micrographs of the \textit{fiddlehead (fdh)} mutant (B) (from Pruiit et al., 2000) and wild-type (A) \textit{Arabidopsis}, showing the fusion phenotype of the inflorescences in the mutant. (Scale bars = 0.5cm.)
1.2.4 Reducing water wettability of plant organs

Due to the hydrophobic nature of the plant cuticle, water on the surface of leaves or other organs often gathers into droplets and easily runs off, rather than forming extended films. An excellent example is the self-cleaning behavior of lotus (*Nelumbo nucifera*) leaves (also called the “lotus effect”). The exceptional water repellency of the lotus leaf relies on several features, such as the robustness and the unique properties of the epicuticular wax, and the unique surface topography (Hans Ensikat, et al., 2011).

The reduced wettability of leaf surface can help to keep stomata unclogged by water and facilitate the uptake of carbon dioxide after rain or during the morning hours following the formation of dew. Furthermore, the hydrophobicity of cuticles may also reduce the likelihood of pathogen infection because fungal spores have fewer opportunities for germination. It has been shown that the presence of a dense layer of epicuticular wax crystals, waxy trichomes and papillose epidermal cells may strongly increase the water repellency of leaf surface.

1.2.5 Protecting against UV light damage

The precise role of the cuticle in providing protection against UV radiation at the plant surface is still not well understood (Zhang et al., 2007, Javelle et al., 2011), although it has been suggested that the light-scattering properties of the cuticular layer may contribute to reducing UV-light absorption.
by the plant (Shepherd and Griffiths, 2006). Some studies have indicated that cuticular phenolic compounds (e.g. cinnamic acids and flavonoids) can absorb or scatter UV light, thereby reducing UV radiation damage to the plant (Solovchenko and Merzlyak, 2003; Kolb and Pfundel, 2005).

1.2.6 Other roles of cuticle in plant development

Some mutants impaired in cuticle biosynthesis exhibit not only a deficiency in those cuticular chemical compounds but also a variety of developmental abnormalities, including embryo/seedling lethality and crinkled organs. This phenomenon suggests that plant cuticle may play other roles in plant development, or that those genes may be indirectly related to other important physiological processes.

Some cuticle-defective mutants are also affected in stomatal density or patterning, or altered in trichome density or its distribution (Kurata et al., 2003; Aharoni et al., 2004; Sturaro et al., 2005). One possible explanation for this is that cuticular permeability changes can alter a physiological signal network which globally regulates water balance.

1.3 Cuticle Monomer Biosynthesis

It is estimated that over half of the fatty acids produced in stem epidermal cells of Arabidopsis thaliana are exported into the cuticular layer for cutin and wax load, underlining the importance of cuticle biosynthesis in
epidermal cell metabolism (Suh et al., 2005). Many biosynthetic enzymes of the plant cuticle have been identified by comparing the lipid profiles of cuticle defective mutants to their wild-types (Neuffer et al., 1997; Pollard et al., 2008; Samuels et al., 2008; Kunst and Samuels, 2009, Javelle et al., 2011). Following a brief summary of current knowledge regarding wax biosynthesis and associated cutin monomer biosynthesis is presented.

1.3.1 Wax biosynthesis

The successive steps of enzymatic reactions in wax biosynthesis in Arabidopsis thaliana are well studied and have been described in several review papers (Nawrath, 2006; Samuels et al., 2008; Dominguez et al., 2011; Javelle et al., 2011). A schematic overview of wax biosynthesis pathways is illustrated in Figure 1.6. In brief, the elongation of C16–C18 fatty acids (FAs) into VLCFAs (C20 to C34) is catalyzed in the endoplasmic reticulum (ER) by a fatty acid elongase (FAE) complex, which is composed of four distinct enzymes: 3-ketoacyl-CoA synthase (KCS), 3-ketoacyl reductase (KCR), 3-hydroxyacyl-CoA dehydratase (HCD) and enoyl-CoA reductase (ECR) (Bach and Faure, 2010). The major wax products are synthesized from VLCFAs via two pathways: the alkane pathway and the wax ester pathway (Kunst and Samuels, 2003). In the alkane pathway, VLCFAs are reduced to aldehydes by an unknown long-chain-fatty-acyl-CoA reductase, and then decarbonylated into odd-numbered alkanes by a fatty aldehyde decarbonylase (CER1).
Figure 1.6 Biosynthetic pathways of major cuticular wax molecules.

CER1: fatty aldehyde decarbonylase; FAE Complex: fatty acid elongase complex; FAR: fatty acid reductase; MAH1: midchain alkanes hydroxylase; WSD1: wax ester synthase/acyl-coa: diacylglycerol acyltransferase
Subsequently, they are synthesized into corresponding secondary alcohols and ketones via a mid-chain alkane hydroxylase (MAH1) enzymatic reaction (Greer et al., 2007; Samuels et al., 2008). In the wax ester pathway, VLCFAs are reduced to primary alcohols by fatty acyl-CoA reductase (FAR), and subsequently condensed with C16/C18 fatty acids into wax esters by wax ester synthase (WSD1) (Rowland et al., 2006; Li et al., 2008).

1.3.2 Cutin monomer biosynthesis

To date, considerable progress has been made in the elucidation of the biosynthetic pathway of cutin monomers and the major enzymes involved in cutin monomer biosynthesis have been identified by forward or reverse genetic analysis and biochemical analysis of the corresponding recombinant proteins (Kolattukudy, 2001; Herecldia, 2003; Pollards, 2008; Reina-Pinto and Yephremov 2009; Pinot and Beisson, 2011). Common cutin monomers of the plant cuticle, such as 10, 16-dihydroxy C16 acid, 9,10,18-trihydroxy C18 acid and 18-hydroxy-9,10 epoxy C18 acid (Fig. 1.2A), are synthesized in the epidermal cells from the corresponding C16 and C18 fatty acids by ω-hydroxylation, in-chain hydroxylation, epoxidation and epoxide hydration. The oxygenation reactions of fatty acids are mainly catalyzed by the enzymes in the cytochrome P450 family (P450s), which represents one of the largest superfamilies of plant proteins (Pinot and Beisson 2011).
It should be noted that most cutin monomer substrates (fatty acids) are first conjugated to acyl-CoA by long-chain acyl-Coenzyme A synthetase (LACS), which is a key activation step for the subsequent oxidation reaction of FAs. Multiple substrate oxidation events may result in a complex pathway, depending on their relative sequence. The simplified biosynthetic steps of the major cutin monomers are depicted in Figure 1.7.

ω-hydroxylation: Three enzymes with alkane 1-monoxygenase activity (CYP86A8, CYP86A2 and CYP86A4) in the CYP86A subfamily, have been confirmed to be involved in the ω-hydroxylation of cutin monomers in *Arabidopsis* (Wellesen et al., 2001; Duan and Schuler 2005; Molina et al., 2008; Pinot and Beisson 2011). It reported that YP86A8 (LCR) can catalyze ω-hydroxylation of fatty acids ranging from C12 to C18:1, but that 9, 10-epoxystearate is not an efficient substrate for this enzyme (Wellesen et al., 2001). In contrast, CYP94C1 has the highest ω-hydroxylase activity with 9,10-epoxystearic acid; meanwhile, it can also catalyze ω-hydroxylation of lauric acid (C12:0) and C18 unsaturated fatty acids (Kandel et al., 2007). It has also been demonstrated that another gene *CYP704B1*, which is essential for pollen exine development of *Arabidopsis*, encodes a ω-hydroxylase that can catalyze ω-hydroxylation of long-chain fatty acids when expressed in yeast cells (Dobritsa, et al., 2009).
Figure 1.7 The biosynthetic pathways of major cutin monomers.
(A) The biosynthesis of C16 cutin monomers. (B) The biosynthesis of C18 cutin monomers. Note: Fatty acids are first activated to Acyl-CoAs, followed by other synthesis steps. HTH: hothead/adhesion of calix edge. Other CYP enzymes in the blue spheres belong to Cytochrome P450 superfamily (abbreviated as CYP), which are found in Arabidopsis.
**In-chain hydroxylation:** It has been suggested that the introduction of in-chain hydroxyl group into ω-hydroxy FAs allows cross-linking between cutin monomers, leading to a reticulation structure and strengthening of the polymeric envelope (Pinot and Beisson 2011). Recently, the *Arabidopsis* gene *CYP77A6*, which encodes an in-chain hydroxylase, was cloned from a mutant lacking nanoridges on the surface of the flowers. Biochemical activity analysis using CYP77A6-expressing yeast microsomes demonstrated that 10, 16 dihydroxypalmitrates can be synthesized by CYP77A6 from 16-hydroxy-palmitate (Li-Beisson et al., 2009).

**Epoxidation:** The 9, 10-dihydroxy FAs are derived from unsaturated FAs (such as oleic acid, linoleic acid) by the epoxidation of their double bonds, followed by the hydroxylation of the epoxide to produce oxygenated FAs (Pinot et al., 1992, 1999; Osman, 1995; Kolattukudy, 2001). It has been reported that CYP77A4 enzyme from *Arabidopsis* is able to synthesize epoxides by epoxidating the double bond of unsaturated fatty acids, after which further synthesis to the corresponding diols is accomplished by an unidentified epoxide hydrolase (Sauveplane et al., 2009).

**Dicarboxylic acid (DCA) synthesis:** DCAs are thought to make a significant contribution to the formation of the cross-linked domain in cutin biosynthesis. The *Arabidopsis* organ fusion mutant *hothead* (*hth*) shows decreased α-, ω-dicarboxylic fatty acid content in the leaf cuticle polyesters, suggesting that HTH may function as an enzyme in ω-oxidation pathway to
oxidize long-chain ω-hydroxyl fatty acids into ω-oxo fatty acids. The HTH protein shows high sequence similarity to long-chain FA ω-alcohol dehydrogenases from *Candida* species, prompting speculation that it may catalyze the next step after ω-hydroxylases in the ω-oxidation pathway (Fig. 1.8) (Kurdyukov et al., 2006).

From research on synthesizing dicarboxylic fatty acid *in vitro*, dodecadioic acid (a diacid) can be synthesized from a 12-hydroxylauric acid substrate by incubating recombinant microsomes of yeasts transformed with CYP94C1 plasmid, but not with a control plasmid. This suggests the possible involvement of the CYP94C1 enzyme in the biosynthesis of diacids in *Arabidopsis* (Kandel et al., 2007). In a prior study, it was found that the enzyme CYP94A5 from *Nicotiana tabacum* can catalyze the oxidation of fatty acids to produce the ω-alcohol, then to the corresponding diacid (Bouquin et al., 2001).

It is worth noting that much of the summarized knowledge of FA oxidation comes from biochemical studies performed after heterologous expression in yeast. Moreover, the activity measurements were performed with free FA, not natural substrates (e.g. acyl-CoAs or glycerolipids etc). Therefore, the exact enzymatic reactions of some oxidation steps of FAs, such as epoxide hydration and oxidation of ω-hydroxyl, may require further elucidation.
1.4 Cutin Monomer Export and Assembly

Based on subcellular localization studies, the majority of biosynthetic enzymes of wax and cutin monomers reside in the ER, suggesting that the final monomers of the plant cuticle are probably synthesized in this compartment (Rowland et al., 2006; Greer et al., 2007; Li et al., 2008; Reina-Pinto and Yephremov 2009). The mechanisms behind the export and assembly of wax and cutin monomers to the outer cuticle remain poorly understood. Several reviews have discussed and proposed the possible transport paths of cuticular lipids (Pollard, 2008; Samuels et al., 2008; Kunst and Samuels, 2009). Here, I will shed light on recent progress in the export and assembly of cutin molecules.

1.4.1 \textit{sn}-2-monoacylglycerol (2-MAG), the major transport substrate and acyl donor in cutin biosynthesis

Cutin monomers must traffic from their synthetic site to the plasma membrane (PM); move across the PM, and cross the polysaccharide rich cell wall in the hydrophilic apoplastic environment, before reaching to their assembly site, the cuticle. Although direct experimental evidence identifying the actual transport substrates of the cutin components is still lacking, indirect evidence suggests that 2-monoacylglycerol (2-MAG) may serve as a transport substrate and acyl donor in the cutin biosynthesis pathway. It has been demonstrated that two acyltransferases, GPAT4 and GPAT6, which are
required for cutin formation in *Arabidopsis*, can esterify acyl groups of FAs predominantly to the *sn*-2 position of G3P, resulting in the *sn*2-MAG as a major product (Yang et al., 2010). The authors speculated that 2-MAG might serve as a cutin precursor for export and assembly.

A detailed biosynthetic pathway of 2-MAG and cutin polymer is illustrated in Figure 1.8. Briefly, ω-hydroxy fatty acid is first activated by long-chain acyl-CoA synthetase (LACS) to produce corresponding ω-hydroxy fatty acyl-CoA (Schnurr et al., 2004), then 2-MAG is synthesized via esterifying the acyl groups of ω-hydroxy FA to the *sn*-2 position of G3P by acyltransferases (GPAT4 or GPAT6) (Yang et al., 2010). At a cutin synthesis site, the acyl group of 2-MAG (acyl donor) is presumably then esterified to the growing cutin polymer.

1.4.2 Export of cutin monomers

**The synthetic site of cutin monomers:** The biosynthesis site of the cutin monomer (2-MAG) remains uncertain, because experimental evidence is still inadequate regarding subcellular localization of two acyltransferases, GPAT4 and GPAT6, which are required for 2-MAG biosynthesis (Yang et al., 2010). GPAT8, the closest homolog of GPAT4, has been demonstrated to be localized in the ER with both N and C termini in the cytosol (Li et al., 2007; Gidda et al., 2009). Moreover, the C-termini of GPAT4 and GPAT6 contain
**Figure 1.8** Cutin polymerization pathway.

- **GPAT**: glycerol phosphate acyltransferase
- **LACS**: long-chain acyl-CoA synthetase

The diagram shows the cutin polymerization pathway involving the following reactions:

1. **LACS**: Long-chain acyl-CoA synthetase acts as an acceptor in the polymerization cycle.

2. **GPAT**: Glycerol phosphate acyltransferase catalyzes the reaction.

3. The polymerized product (cutin) can act as an acceptor in new polymerization cycles.

The chemical reactions involved are:

- **10,16-diOH Palmitate (FAs)**
- **10,16-diOH Palmitate-CoA (Acyl-CoA)**
- **glycerol-3-phosphate (G3P)**
- **2-MHG (2-MAG, Acyl Donor)**
- **Acyl Acceptor**

The polymerized product (cutin) is shown in the diagram.
diverged-type dilysine motifs (–KXKXXX–COOH and –KKXK–COOH, respectively). Accordingly, a reasonable hypothesis is that GPAT4 and GPAT6 reside in the ER, although whether the two diverged dilysine sequences function as ER retrieval signals remains to be experimentally tested.

**ABC transporter:** The cutin monomers must be transported from the synthesis site to the apoplastic assembly site in the plant cuticle. During this process, they must cross the PM, a process that is most likely mediated by ATP-binding cassette G subfamily transporters (ABCG transporter) (Bird et al., 2007; Verrier et al., 2008; Panikashvili and Aharoni 2008; Bessire et al., 2011; Chen et al., 2011; Panikashvili et al., 2011). The ABC transporter subfamily G includes both white-brown complex (WBC) ‘half-size transporters’ and pleiotropic drug resistance (PDR) ‘full-length transporters’, with more than 40 members in both *Arabidopsis* and rice (Verrier et al., 2008). ABCG family half-transporters (WBC), with 28 genes annotated in the *Arabidopsis* genome, must dimerize to form a full-length, functional ABC transporter (Verrier et al., 2008). *abcg11/wbc11/desperado/cof1*, a well-characterized ABC half-size transporter mutant, displays an array of cuticle defects and a dramatic reduction in the levels of waxes and cutin monomers (Bird et al., 2007; Luo et al., 2007; Panikashvili et al., 2007; Ukitsu et al., 2007; Panikashvili et al., 2010; McFarlane et al., 2010). In contrast, *abcg12/cer5*, another ABCG half-size transporter mutant, is phenotypically normal except for having abnormally
glossy stems, which reflects a reduction of wax components in the cuticle (Rashotte et al., 2001; Pighin et al., 2004). In an in-vivo interaction experiment of the two half-size transporters using bimolecular fluorescence complementation (BiFC), it has been reported that ABCG11/ABCG12 heterodimers and ABCG11 homodimers can be detected in the PM of epidermal cells, while ABCG12 homodimers cannot. The protein trafficking assays showed that ABCG11 can travel to the plasma membrane, while, ABCG12 is retained in the ER in the case of ABCG11 knockout. This suggests that ABCG11 is capable of forming flexible dimer partnerships, but that ABCG12 is only capable of forming a dimer with ABCG11 (McFarlane et al., 2010).

Another Arabidopsis half-size transporter gene ABCG13 was recently characterized (Panikashvili et al., 2011) and it was reported that loss of ABCG13 function resulted in cuticle-related phenotypes and caused a significant reduction in levels of cutin monomers in the flowers. This suggests that ABCG13 is probably required for Arabidopsis flower cutin monomer secretion.

Evidence is accumulating that full-length subfamily G transporters, known as pleiotropic drug resistance (PDR), also are probably associated with cutin monomer export. The permeable cuticle 1 (pec1) Arabidopsis mutant, caused by the knockout of the ATP Binding Cassette G32 (abcg32) gene, displays a highly permeable cuticle phenotype. The ABCG32 full-length transporter
shows polar localization in PM regions near the plant surface. Further, cutin profiling data reveal that typical cutin monomers (e.g. ω-hydroxy fatty acids) in pec1 flowers, are reduced to 40% of wild-type levels, suggesting that ABCG32 likely exports cutin precursors to the plant cuticle (Bessire et al., 2011). A similar result is also found in other plants: the eibi1 mutant of wild barley, which carries a spontaneous mutation in an ATP-binding cassette (ABC) subfamily G full transporter (HvABCG31), displays a phenotype of cutin disorganization and a low capacity to retain leaf water. Quantification of cuticle components showed that levels of the major cutin monomers in the eibi1 mutant are reduced to about 50% of those in wild barley, but that there is little difference in the major wax component (1-hexacosanol) between the two lines. This result suggests that HvABCG31 is likely required for cutin load (Chen et al., 2011).

**Possible role of transfer proteins:** Lipid transfer proteins (LTPs) are abundant in the *Arabidopsis* genome, with about 70 members in total. It has been proposed that LTPs may play a role in the transfer of cuticle components during the cuticle development (Yeats and Rose, 2008). The first convincing evidence of the role of LTPs in the export of cuticular components was from a study of the *ltpg* mutant of *Arabidopsis* (Debono et al., 2009). The *LTPG* gene encodes a glycosylphosphatidyl-inositol-anchored lipid transfer protein. Mutant lines with decreased *LTPG* expression exhibit a reduced total wax load (mainly alkanes) in the cuticle, indicating that LTPG likely contributes to
cuticular wax deposition (Debono et al., 2009, Lee et al., 2009). However, it remains unclear whether LTPG delivers lipids across the cell wall to the cuticle, because LTPG is a PM-bound protein (Kunst and Samuels, 2009).

It is thought that LTPs are probably not capable of facilitating intracellular lipid trafficking due to their extracellular localization feature (Yeats and Rose, 2008). Therefore, the trafficking route and molecular mechanisms by which cuticle lipid precursors move from the ER to the PM in the cytosol remain unknown. Acyl-CoA-binding proteins (ACBPs) have been proposed as candidates for intracellular lipid transfer in plant cells (Xiao and Chye 2009). Six members of the ACBP group have been identified from Arabidopsis, and some of them reside in cytoplasm. However, no direct or indirect evidence to date shows a relationship between Acyl-CoA-binding proteins (ACBPs) and cuticle lipid loading.

Unlike cuticular wax molecules, 2-MAGs have significant aqueous solubility (Yang et al., 2010), and this property may substantially facilitate the movement of 2-MAG in the cytosol or in an extracellular aqueous matrix. Here, it is speculated that the movement of cutin monomers (2-MAGs) is mediated by passive diffusion in an aqueous matrix, although the possibility that the traffic of cutin precursors is facilitated by a protein binding process cannot yet be rejected.
1.4.3 Assembly of cutin monomers

Cutin is a nonlinear, or cross-linked, polymer synthesized from cutin monomers. However, the mechanisms that mediate cutin polymerization have been undetermined until recently. *In-vitro* cutin polymerization by crude plant enzyme preparations was first reported in 1974: insoluble cutin biopolymer can be synthesized from 16-hydroxypalmitic acid, and 10, 16-dihydroxypalmitic acid (cutin monomers), using extracts from young *Vicia faba* leaf epidermal tissues with adenosine triphosphate and coenzyme A as the required cofactors (Croteau and Kolattukudy 1974). It was suggested that an unknown acyltransferase can then transfer the hydroxyacyl moieties of cutin monomers to the growing cutin polymer. Following this hypothesis, a 17 kDa protein was isolated by native PAGE electrophoresis from a crude leaf epidermis extract of *Agave americana*. The acyl-CoA:cutin transferase activity of this purified protein was tested *in vitro* using chemically synthesized oligohydroxy fatty esters as acceptors and labeled palmitoyl-CoA and oleoyl-CoA as acyl donors. The corresponding interesterified compounds were obtained from this experiment (Reina and Heredia, 2001). The 17 kDa protein was partially sequenced, but the sequence data presented in this paper were not informative; and the corresponding gene was not cloned. In another study by same research group, the gene *AgaSGNH*, encoding a SGNH-hydrolase, was isolated from the cDNA library of young leaf epidermis of *A. americana* using degenerated PCR primers designed according to the N-terminal
sequence of that purified 17 kDa protein (Reina and Heredia, 2001; Reina et al., 2007). It was demonstrated that the AgaSGNH protein was localized in the outer cell wall of the epidermal cells and had an acyltransferase activity, leading investigators to speculate that the protein is probably involved in plant cutin biosynthesis (Reina et al., 2007). In this paper, the authors mentioned that the amino terminal peptide sequence chosen to design the cloning primers was not present in the PCR fragment of the AgaSGNH cDNA; hence the function of AgaSGNH protein remains somewhat uncertain as there was no apparent association between the AgaSGNH gene and the purified 17 kDa protein.

The bodyguard (bdg) mutant of Arabidopsis exhibits a disorganized cuticle structure, but unexpectedly accumulates significantly more residual bound lipids (mainly cutin) and waxes per unit of leaf surface area. The BDG protein, which is located in the outermost cell wall of epidermal cells, belongs to a newly identified class of the α/β-hydrolase superfamily. The authors hypothesized that BDG may act directly as an extracellular synthase for cuticle formation (Kurdyukov et al., 2006). However, the function of this enzyme remains obscure, because no biochemical activity of BDG protein has been experimentally tested.
1.4.4 Proposed pathways of cutin monomer export and cutin synthesis

An overview of cutin biosynthesis, including the possible synthetic site, proposed transfer pathways and assembly site of cutin monomers is presented in Figure 1.9. In brief, sn-2 MAGs are presumably synthesized in the ER by the acyltransferases GPAT4 or GPAT6. Water soluble 2-MAG molecules likely move near the PM by passive diffusion, then are channeled through the PM by ABC-G family transporters, and finally move across the cell wall by passive diffusion or some other more coordinated mechanism.

Other possible transfer mechanisms of cuticle precursors, such as Golgi-mediated vesicular traffic through the secretion pathway and oleophilic droplet transport pathway have been proposed in several review papers (Kunst et al., 2006; Panikashvili and Aharoni 2008; Samuels et al., 2008). However, those proposed export pathways may exist for the transport of cuticular waxes but there is no solid evidence to substantiate that cutin monomers may be transferred via those pathways. The possible transport pathways for cuticular lipid are also illustrated in Figure 1.9 for reference (shown with dotted lines).
Figure 1.9  Biosynthesis and transport of cutin monomers.

In the ER, ω-hydroxy fatty acids are synthesized by several enzymatic oxidation reactions (CYP86A, CYP77A and HTH etc.), next are esterified to glycerol-3-phosphate by GPAT enzymes to synthesize 2-MAGs. The transport path of the cutin monomers (2-MAGs) in the cytoplasm and extracellular matrix remains unclear. Current evidence suggests that ABC transporters are likely involved in channeling cutin monomer across the PM. Several combinations of ABCG transporter pathways are illustrated in the diagram. Other proposed transport pathways of cuticular lipids such as oleophilic bodies and secretory vesicles, are still unresolved due to the lack of reliable evidence, and are drawn in dotted lines for reference.
1.5 Transcriptional Regulation of Cutin Biosynthesis

Knowledge of the transcriptional regulation of cutin-related metabolic pathways during plant development remains relatively sparse. The *WAX INDUCER 1* (*WIN1/SHN1*) gene, which encodes a member of the AP2/ERF (ethylene response factor) transcription factor family, can trigger wax production, thus enhancing plant drought tolerance (Aharoni et al., 2004; Broun et al., 2004). Further analysis has shown that *WIN1/SHN1* can also modulate cutin biosynthesis: overexpression of the *WIN1/SHN1* gene increased the cutin load on plant organs, while its down-regulation has the opposite effect (Kannangara et al., 2007). Regulatory network analysis has shown that the induction of *WIN1/SHN1* causes a rapid increase in the expression of some genes involved in cutin biosynthesis, including CYP86A7, CYP86A4, a GDSL-motif lipase/hydrolase, HTH-like and LACS2. In a chromatin immune-precipitation (ChIP) experiment, the LACS2 promoter was detected in *WIN1/SHN1*-binding genomic fragments, suggesting that LACS2 is likely to be directly targeted by this transcription factor (Kannangara et al., 2007). The result of co-silencing all three *SHN/WIN* clade members using an artificial microRNA approach demonstrated that the transcription factors act directly on downstream cutin and cell wall–modifying genes and broadly control floral organ development, specifically affecting cuticle assembly and cell wall remodeling (Shi et al., 2011). Nevertheless, current knowledge of the
modes of action of the SHN1/WIN1 transcription factor and involvement in particular pathways or processes is still limited.

The *serrate* (*se*) gene of *Arabidopsis* which encodes a protein of RNA-processing multi-protein complexes, can mediate the transcription of some genes associated with cutin and wax biosynthesis in a compensatory manner in several cuticle mutants, such as *lacerata* (*lcr*, CYP86A8), *fiddlehead* (*fdh*, *kcs10*) and *bodyguard* (*bdg*) (Voisin et al., 2009). Mutations in those genes cause a complex developmental syndrome that is characterized by ectopic organ fusions and leaf deformations. Unexpectedly, the mutants over-accumulate major cutin monomers in leaves. A genome-wide gene expression analysis by microarray showed that the range of genes involved in cuticle biosynthesis are coordinately up-regulated in all three cuticular mutants, suggesting that those mutants can alleviate the functional disorder of the cuticle by reinforcing their defense network. The *serrate*, gene was identified as a mediator (or enhancer) among putative enhancers and suppressors by meta-analysis of microarray data and its role as a mediator has been confirmed by the experimental result that the *se lcr* and *se bdg* double mutants can eradicate the typical phenotypes of *lcr* and *bdg*, such as organ fusions and severe leaf deformations (Voisin et al., 2009).
1.6 Tomato, an Attractive Model System for fruit Cuticle Studies

*Arabidopsis thaliana* has been widely used as a robust model for cutin biosynthesis studies for more than a decade, and many cuticle-associated mutants have been identified and cloned from this model plant (Nawrath, 2006; Pollard et al., 2008; Samuels et al., 2008; Javelle et al., 2011). However, high amounts of the C18:2 α, ω-dicarboxylic acid (DCA) cutin monomer (more than 50%) and glycerol are present in the cutin of *Arabidopsis*, suggesting that DCAs and glycerol molecules are presumably directly esterified together to produce atypical DCA–glycerol cutin polymers (Bonaventure et al., 2004; Franke et al., 2005; Pollard et al., 2008). Therefore, *Arabidopsis* is not a good representative model for cutin biology (Dominguez et al., 2010). In addition, the cuticle of *Arabidopsis* is thin and extremely delicate, increasing the difficulty of cuticle isolation and handling (Franke et al., 2005; Isaacson et al., 2009). Tomato fruit, on the other hand, offers an ideal alternative model system for cuticle studies. In tomato fruit cuticle, 9(10), 16-dihydroxy hexadecanoic acid is the most abundant cutin monomer (accounting for about 80% of total), which is also one of the typical cutin monomers (ω-hydroxy fatty acids) in many plants (Isaacson et al., 2009). Tomato fruit cuticles are relatively thick, easy to isolate and are astomatous, thereby providing intact cuticular membranes, which will help with functional characterizations of
cuticle permeability and pathogen infection (Vogg et al., 2004; Leide et al., 2011).

In the last decade, extensive tomato sequence data, such as numerous molecular markers and massive EST sequences, have been collected (http://solgenomics.net). This has culminated in a high-quality draft of the tomato genome sequence, and a gene annotation built by the International Tomato Genome Sequencing Project. Mutational approaches have been highly exploited in tomato genetic and molecular research. Several tomato mutant collections have been generated by fast neutron or EMS mutagenesis in different research labs (Menda et al., 2004; Minoia et al., 2010; Saito et al., 2011). In addition, tomato fruit cuticle have been studied in recent years, leading to the accumulation of a useful information regarding fruit cuticle biology (Leide et al., 2007; Mintz-Oron et al., 2008; Heredia et al., 2008; Buda et al., 2009; Yeats et al., 2010; Kosma et al., 2010; Leide et al., 2011; Matas et al., 2011).

Tomato is one of the world’s most important vegetable crops and has emerged as the primary model system for studying climacteric fruit development, ripening and metabolism study. Studies to date have suggested that fruit cuticle is one of the key contributing factors influencing tomato fruit storability and shelf life (Saladie et al., 2007; Kosma et al., 2010). The elastic properties of plant cuticular membranes may play an important role in maintaining fruit integrity and reducing fruit cracking (Bargel et al., 2005;
Bargel and Neinhuis, 2005; Dominguez et al., 2011). Moreover, it has been suggested that the variation in fruit cuticle structure or composition may influence other important fruit traits, such as resistance to desiccation and microbial infection.

Considering the advantages such as typical cutin composition profile, the economic and scientific importance, and the abundant genetic information and materials, the tomato is an ideal model system for plant cuticle biology studies.
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CHAPTER TWO

Map-Based Cloning of Three Fruit Cutin-Deficient Mutants of Tomato

2.1 Introduction

Biologically and commercially important features of plant fruit cuticles include their structure, composition and thickness, all of which may influence many important fruit traits, such as storability/shelf life, surface glossiness, and the capability of resistance to desiccation, microbial infection or cracking (Bargel et al., 2006; Bargel and Neinhuis, 2005; Kosma et al., 2010). Despite the fundamental importance of fruit cuticle, our knowledge of its synthesis and metabolism remains limited. A better understanding of the genetic and molecular mechanism underlying fruit cuticle formation in a model species is crucial for the genetic improvement of fruit crops. In this regard tomato, one of the most important vegetable and fruit crops, is an emerging model for fruit cuticle biology, as its fruit cuticle is compositionally typical of most plant cuticles, thick, astomatous and easily isolated compared to that of the experimental model plant Arabidopsis thaliana.
To date, most knowledge about cuticle biology has been obtained from the genetic and biochemical characterization of cuticle-related mutants and more than 50 genes involved in cuticle biosynthesis have been identified, mostly from Arabidopsis, maize or rice (Pollard et al., 2008; Samuels et al., 2008; Kunst and Samuels, 2009; Javelle et al., 2010).

Forward genetic approaches are valuable tool for identifying gene function or establishing a connection between the important phenotype and the genes. Screening genetic mutants from a mutagenesis population is the primary step for forward genetic analysis. Several tomato mutant collections using EMS or irradiation mutagenesis approaches have been released by different research labs (Menda et al., 2004; Minoia et al., 2010; Saito et al., 2011) (http://zamir.sgn.cornell.edu/mutants/; http://tgrc.ucdavis.edu/; http://tomatoma.nbrp.jp/index.jsp). In order to identify the genes involved in fruit cuticle biosynthesis, three fruit cuticle mutants obtained from the ‘Genes that Make Tomatoes’ collection (Menda et al., 2004) were chosen for gene function studies using map-based cloning strategy. The mapping results of these three genes are reported here.

Cloning of these three tomato genes was a substantial undertaking and took place at a time when there was no draft tomato genome sequence available. Therefore, this gene cloning was accomplished by a research group. Dr. Tal Isaacson initiated the mapping studies and did much of the phenotype analysis for the cutin-deficient mutants. Yonghua He did most genotyping
work for the cloning of cd1 and cd2, as well as the part of cd3 genotyping. Dr. Lingxia Zhao performed some genotyping for the fine-mapping of cd3 gene and also the part of cd1 genotyping.

2.2 Material and Methods

2.2.1 Tomato mutants, cultivars and growth conditions

Three tomato mutant lines e4247m1, e4393m2 and n3056m1 were derived from an EMS or fast neutron mutagenized population under the genetic background of tomato cultivar M82 (Menda et al., 2004; http://zamir.sgn.cornell.edu/ mutants/). These three cuticle mutants shared a similar phenotypic trait in the form of a dramatically reduced cuticular cutin load. As such, while they were named cuticle mutants 14, 15 and 20. They were later renamed cutin-deficient 1 to 3 (cd1, cd2, cd3), respectively. The basic information about the mutants is listed in Table 2.1 and photographs of the mutant fruits from the ‘Genes that Make Tomatoes’ website are shown in Figure 2.1.

The fruits of three cutin-deficient mutants have a highly glossy appearance and a ‘rubbery’ surface texture with abnormal fruit color (Fig. 2.1 and Fig. 2.2), compared with those of wild-type M82, but no visible phenotype in other organs.
**Table 2.1** Background information related to the three *cutin-deficient* mutants

<table>
<thead>
<tr>
<th>Mutant lines</th>
<th>Mutagenesis</th>
<th>Phenotype description</th>
</tr>
</thead>
<tbody>
<tr>
<td>e4247m1 (CM14, cd1)</td>
<td>EMS</td>
<td>Waxy epidermis</td>
</tr>
<tr>
<td>e4393m2 (CM15, cd2)</td>
<td>EMS</td>
<td>Colorless waxy epidermis; orange-tinge flesh; pink-tinge epidermis</td>
</tr>
<tr>
<td>n3056m1 (CM20, cd3)</td>
<td>Fast neutron</td>
<td>Gold cuticle</td>
</tr>
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</table>

To assess whether the glossy fruit phenotypes of the three putative cuticle mutants were associated with quantitative or qualitative differences in wax or cutin content, the composition of isolated fruit cuticles from the three mutants and wild-type M82 at small green, mature green and red ripe stages were analyzed by GC-MS. The most striking result was that all three mutant
lines had a significantly lower level of total cutin per unit fruit surface area compared to M82, which indicated the severe cutin deficiency in these mutants. The fruits of the cutin-deficient mutants had normal levels of wax on a mass per area basis, but the wax compositions among mutant lines were somewhat different from wild type and from each other (Isaacson et al., 2009). Herein, the total cutin and wax load of fruit from the three mutants were depicted in Figure 2.3 for reference. The light microscope images of the fruit cutin layers of the three mutants and wild-type M82 stained by Sudan IV are shown in Figure 2.3A.

Three cutin-deficient mutants were back-crossed with M82 and crossed with each other for alleleism analyses. The segregation ratios of the glossy
Figure 2.3  Total fruit cutin monomer (A) and wax (B) loads of cd1, cd2 and cd3 mutants and M82 in mature green stage.
(Adopted from Isaacson et al., 2009. Scale bars in panel A = 20 μm)
traits in the progeny indicated that the mutations occurred at three distinct gene loci. Moreover, the traits were inherited in a recessive mode in all three mutant lines. 

**Growth conditions:** Plants were grown in a greenhouse in Ithaca, New York, with a photoperiod of 16h/8h (day/night), using standard practices.

### 2.2.2 Development of mapping population

Three *cutin deficient* mutants were crossed with the wild species *S. pimpinellifolium* (LA1589) to produce F₁ progenies. Seeds of the F₂ population were harvested from self-crossed F₁ plants of each population (Fig. 2.4).

![Diagram](image)

**Figure 2.4** Development of F₂ mapping population of *cutin-deficient* mutants.
2.2.3 Marker design and DNA extraction

**Marker development and design:** Genetic markers designed for tomato TA209/S. pimpinellifolium mapping population in Dr. S. Tanksley’s lab (Cornell University) were validated for the M82/S. pimpinellifolium population. For genome-wide genotyping, about 60 genetic markers were chosen that are distributed evenly across the 12 chromosomes with an interval about 20~30cM. The CAPs and dCAPs markers for gene fine mapping were designed according to the DNA sequence polymorphism by CAPS Designer (http://solgenomics.net/tools/caps_designer /caps_input.pl) and dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html). Descriptions of the genetic markers including marker name, location, restriction enzymes and marker quality are listed in Appendix I.

**DNA extraction:** For each mapping population, young leaf samples from the 94 F₂ plants plus 2 parents were collected. Genomic DNA was extracted according to the method described by Fulton et al. (1995).

2.2.4 Genotyping and phenotyping

**Genotyping:** PCR reactions were performed following standard protocols. PCR products were digested with appropriate restriction enzymes then fractionated on 4% agarose gels. For the first mapping population, 94
cd2 samples were genotyped with the 60 markers. Since it was determined that it was not necessary to screen with 94 samples for each population. To reduce the workload, 96 DNA samples from three mapping populations (cd1, cd3 and hcr1, 32 samples each) were pipetted into a new plate, then genotyped with 48 markers.

**Phenotyping:** The phenotypes of three cutin-deficient mutants were scored by assessing fruit cuticle thickness using the enzymatic digestion method described in Isaacson et al. (2009).

### 2.2.5 Mapping data analysis

Genotype and phenotype data were analyzed using Qgene software 4.0 ([http://coding.plantpath.ksu.edu/qgene/](http://coding.plantpath.ksu.edu/qgene/)) (Joehanes and Nelson, 2008). The QTL analysis was performed with two regression models: Simple Interval Mapping (SIM) and Composite Interval Mapping (CIM-LS) with cofactor selection.

### 2.3 Results

#### 2.3.1 mapping the cd1 locus

30 plants of the F2 population were phenotyped for cuticle thickness and then genotyped with 48 markers. The QTL map of cd1 population is
shown in Figure 2.5. A substantial QTL (LOD = 12) was located near the north end of chromosome 11 and the CD1 gene was located between marker TG497 (11.001) and T1657 (11.009).

Figure 2.5  QTL map in the cd1 mapping population
(A) QTL analysis by Simple Interval Mapping (SIM) model.
(B) QTL analysis by Composite Interval Mapping (CIM-LS) with cofactor maker (with blue background) selection.
   Red line – the value of log-likelihood ratio (LOD) score
   Green line – LOD threshold
(The number in parentheses indicates the marker location in cM.)
For fine mapping, 880 F$_2$ plants from the cd1 population were screened for recombinants with markers TG497 (11.001) and At5g04420 (11.015). A total of 105 recombinants were identified and their cuticle thickness phenotypes were scored. The recombinants were genotyped using newly designed CAPS (or dCAPS) markers. The phenotypic and genotypic data of important recombinants are shown in Table 2.2 and 2.3 in Appendix II.

The location of the cd1 gene was first narrowed down to a 3.22 Mbp tomato WGS scaffold SL1.03sc01386, (corresponding to SL2.40sc03748 in version 2.3 of the ITAG tomato genome annotation, http://solgenomics.net) and subsequently to a 61 kb region between markers P120 and P140 (Fig. 2.6A). The cd1 gene showed perfect co-segregation with marker T1968 (11.005) in all recombinants. Six putative genes were annotated in this 61 kb region and a GDSL-motif lipase/hydrolase gene (SGN-U585129) was chosen as the most promising candidate because it showed very high predicted amino acid sequence similarity (71%) to AgaSGNH, a protein that has been associated with cutin load in the leaves of Agave americana (Reina et al., 2007). A genomic sequence comparison of the candidate gene was performed between M82 and cd1 mutant and a DNA polymorphism was found in the last exon of the gene. Specifically this comprised a ‘G to A’ nucleotide substitution that is predicted to introduce a stop codon (TAG) in the last exon of the cd1 gene, the consequence of which is a predicted truncation of the last 41 amino acids, including 2 residues of the catalytic triad (Fig. 2.6B).
Figure 2.6  Mapping of the cd1 locus.  (A) Schematic diagram showing the mapping of CD1 to chromosome 11 between the markers P120 and P140.  (B) CD1 gene model.  The nucleotide substitution G to A introduces a stop codon (TAG) in the last exon, leading to the truncation of the last 41 amino acids including 2 residues of the catalytic triad.
Thus, a likely functional mutation associated with the \textit{cd1} phenotype was identified. The chromosomal position of \textit{CD1} is SL2.40 ch11:1004362…1007293. The Genbank accession number for \textit{CD1}: JF968592

The \textit{CD1} gene is predicted to encode a 362 amino acid protein (Solyc11g006250.1.1). The BLAST search showed that CD1 contains a SGNH plant lipase like domain (with an E-value = 2.73e-101), and corresponds to a hydrolases in plant specific subfamily of the SGNH-family. It has been suggested that this class of protein is involved in a lipid metabolic process with a hydrolase activity.

\subsection*{2.3.2 mapping \textit{cd2} locus}

94 plants from the \textit{cd2} F$_2$ population were genotyped using 60 markers across whole tomato genome and their fruits were phenotyped for cuticle thickness. QTL analysis of the \textit{cd2} population showed a sharp peak (LOD >300) on chromosome 1 between the two CAPS markers T1162 (1.059) and At4g01210 (1.081) (Fig. 2.7). More than 1,200 plants of the \textit{cd2} F$_2$ population were screened for recombinant events using the two above-mentioned markers. A total of 105 recombinants were identified and their cuticle phenotypes were scored. The phenotypic and the genotypic data of the recombinants are shown in Table 2.4 and 2.5 in Appendix II.

The \textit{cd2} gene co-segregated with marker 14A17sp6 in this mapping population. At the time of the screen, no BAC or other DNA sequence
information was available around the region, other than several markers and BAC end sequences. A BAC hybridization screen was therefore carried out.

**Figure 2.7** QTL map in the cd2 mapping population.

(A) QTL analysis by Simple Interval Mapping (SIM) model.

(B) QTL analysis by Composite Interval Mapping (CIM-LS) with cofactor maker (with blue background) selection.

- Red line – the value of log-likelihood ratio (LOD).
- Green line – LOD threshold (>3).

(The number in parentheses indicates the marker location in cM.)
using the DNA markers 14A17sp6 and TG59 as hybridization probes. Nine BACs were identified using the probes and BAC C01SLm0026F20 (130 Kb) was sequenced.

New markers were designed according to the BAC sequence and the genomic region containing the \textit{cd2} gene was further narrowed down to a region of approximately 8 kb around marker 14A17sp6 (Fig. 2.8A), which contained only one putative gene. A DNA polymorphism was found by genomic sequence comparison between \textit{cd2} and M82, corresponding to a ‘G to A’ mutation in the last exon of the \textit{cd2} gene (Solyc01g091630.2.1:31…3021). This is predicted to result in an amino acid change from a glycine to an arginine at position 736 of the protein (Fig. 2.8B), which likely represents the functional mutation associated with \textit{cd2} phenotype. The chromosomal position of \textit{CD2} is: SL2.40ch01: 76982077…76988233 and the Genbank accession number for \textit{CD2} is GQ222185.

The BLAST search result indicated that the \textit{CD2} gene has high similarity (Identities = 68%, E-value = 3.1e-273) to \textit{HDG1} gene of \textit{Arabidopsis} (AT4G00730, \textit{HOMEODOMAIN GLABROUS 1}). The \textit{CD2} encodes a predicted homeobox-leucine zipper family protein, with 821 amino acids (Solyc01g091630.2.1). The protein contains START_ ArGLABRA2_ like domain (E-value: 2.34e-96) and has sequence-specific DNA binding transcription factor activity.
Figure 2.8  Mapping of the cd2 locus.  (A) Schematic diagram showing the mapping of cd2 to an 8 kb region around marker 14A17sp6 on chromosome 1.  (B) CD2 gene model.  A 'G to A' mutation in the last exon of the HD-Zip IV gene results in an amino acid substitution from glycine to arginine at position 736.
2.3.3  cd3 locus mapping

A total of 47 F₂ plants in the cd3 population were phenotyped for their fruit cuticle thickness. Out of the 47 F₂ plants, a total of 30 (13 with a thin cuticle and 17 with normal cuticles) were genotyped using 48 markers across the genome. The QTL analysis showed that the cd3 gene was located near the south end of chromosome 8, with a QTL peak (LOD =9) between the markers TG 294 (8.087) and U233990 (8.0685) (Fig. 2.9).

Recombinants were screened using two markers C2_At4g11560 (72cM) and TG294 (87cM). The genomic region containing cd3 gene was first narrowed down to a BAC contig (C08.18_contig45) and further fine mapping localized it within a region of approximately 90 kb (Fig. 2.10A). A predicted CYP86A gene in the region was chosen as the most promising candidate as a homolog in Arabidopsis has been shown to be involved in ω-hydroxylation of fatty acids (Wellesen et al., 2001; Rupasinghe et al., 2007). Genomic sequence comparison of this candidate gene between M82 and cd3 showed that a small DNA fragment (1.1 kb, from chromosome 7) is inserted into the exon of this CYP86A gene, leading to the truncation of the last 153 amino acids from its protein (Fig. 2.10B), which likely explains the functional mutation associated with cd2 phenotype. The chromosomal position of CD3 is: SL2.40ch08:61445543…61448207.
Figure 2.9  QTL map in the *cd3* mapping population

(A) QTL analysis by Simple Interval Mapping (SIM) model.

(B) QTL analysis by Composite Interval Mapping (CIM-LS) with cofactor maker (with blue background) selection.

Red line – the value of log-likelihood ratio (LOD) score
Green line – LOD threshold (>3)

(The number in parentheses indicates the marker location in cM.)
Figure 2.10  Mapping of the cd3 locus.  (A) Schematic diagram showing the mapping of cd3 to BAC contig_45.  (B) Gene model of CD3.  A small DNA fragment (from Chr. 7) was inserted into the exon of a CYP86A subfamily gene at Chr. 8, leading to the truncation of the last 153 amino acids from the protein.
The CD3 gene contains only one exon and encodes a 554 amino acids protein (Solyc08g081220.1.1) in the CYP86A subfamily of the cytochrome P450 family. The CD3 protein displays high amino acid sequence similarity with Arabidopsis CYP86A8 (70% identity, P= 8.6e-213) and CYP86A2 (68% identity, P= 2.3e-212), suggesting that CD3 has alkane 1-monoxygenase activity and functions as an enzyme for ω-hydroxylation of fatty acids in the cutin biosynthesis pathway.

2.4 Discussion

The possible functions of three cutin deficient genes in cutin biosynthesis pathway the will be discussed here.

The CD1 gene: The cutin deficient 1 (CD1) gene has been intensively studied by our research group, and its function clarified. It was predicted that the CD1 protein belongs to the GDSL-motif lipase/hydrolase family (Fig. 2.11), with an acyltransferase activity, acting on ester bonds. Immunolocalization analysis indicated that the CD1 protein is almost exclusively localized in the tomato fruit cuticle. Biochemical studies showed that the recombinant CD1 protein has acyltransferase activity, with a preference for 2-MAG as an acyl donor. These results suggested that CD1 catalyzes cutin biosynthesis by successively esterifying the hydroxyacyl group of 2-MAG to the hydroxyl group of growing cutin polymer at the site of cutin formation (unpublished).
In addition, free 2-mono (10, 16-dihydroxyhexadecanoyl) glycerol (2-MHG), a 2-MAG species converted from the predominant cutin monomer of tomato (10, 16-diOH hexeadecanoic > 80%), accumulates in cd1 fruit cuticle (unpublished). This result reinforces the notion that 2-MAG is a putative monomeric cutin precursor, as well as a transport substrate for cutin monomer movement.

Figure 2.11  The alignment of CD1 sequence with the homolog in Arabidopsis. Score = 546 bits (1406), Expect = 5e-160, Method: Compositional matrix adjust. Identities = 256/350 (73%), Positives = 303/350 (87%). AT5G33370 = GDSL-like Lipase/Acylhydrolase superfamily protein.
**The CD2 gene:** The CD2 protein possibly resides in nuclear lamina, as predicted by Subnuclear Compartments Prediction System (Version 2.0) (Lei and Dai, 2005; http://array.bioengr.uic.edu/subnuclear.htm), ESLpred (Bhasin and Raghava, 2004; http://www.imtech.res.in/raghava/eslpred/submit.html) and NucPred (Brameier et al., 2007; http://www.sbc.su.se/~maccallr/nucpred/). A BLAST search showed that the CD2 protein belongs to the IV group of the homeodomain-leucine zipper family (HD-Zip IV) (Fig. 2.12), with two predicted functional domains: a ‘homeodomain’ DNA binding domain and a ‘StAR (steroidogenic acute regulatory protein) related lipid transfer (START)’ domain.

HD-Zip proteins, being transcription factors unique to plants, are encoded by more than 25 genes in *Arabidopsis*, of which 16 members are classified in the HD-Zip IV group (Elhiti and Stasolla, 2009). Several members of the HD-Zip IV group are often expressed in outer cells of plant organs and are considered to regulate epidermis-related biological processes, such as epidermal fate, trichome formation, anthocyanin accumulation and cuticle biosynthesis (Kubo et al., 1999; Otsuga et al., 2001; Abe et al., 2003; Javelle et al., 2010). The phenotype characterization and the gene cloning of the cd2 mutant in our lab made the first direct link between HD-ZIP IV transcription factors and cutin biosynthesis (Isaacson et al., 2009; Javelle et al., 2010).
**Figure 2.12** The alignment of CD2 sequence with an *Arabidopsis* homolog.

Score = 1100 bits (2845), Expect = 0.0, Method: Compositional matrix adjust. Identities = 572/840 (68%), Positives = 662/840 (79%).

ANL2 = AT4G00730, a homeodomain protein in the HD-GLABRA2 group.
Recently, more evidence about the involvement of the HD-ZIP IV gene in the regulation of cutin biosynthesis was obtained from overexpression study of the maize *OCL1* gene. Phylogenetic analysis revealed that the maize OCL1 protein and tomato CD2 protein are clustered in the same clade of the HD-ZIP IV family, suggesting a possible function of OCL1 in the regulation of cutin or wax biosynthesis in maize (Javelle et al., 2010). Fourteen genes that are up- or down-regulated in transgenic maize plants over-expressing *OCL1* were identified by a microarray analysis. These target genes are involved in several physiological processes, such as lipid transfer, cuticle biosynthesis, envelope-related functions and plant defense (Javelle et al., 2010). Interestingly, five of the up-regulated genes shared similar annotation related to lipid transport, including type-2 lipid transfer protein (MZ00031783), SEC14/phosphatidylinositol transfer protein (MZ00031955) and three of the ABC transporters in the WBC11/ABCG11 clade. This finding provided useful clue for the future study of the CD2 transcription factor in tomato fruits.

The *cd2* mutants were phenotypically normal except for their highly glossy fruit surface. The fruit cuticle of *cd1* mutant had a striking reduction (98%) in cutin content, but no significant difference could be detected in wax content, suggesting that this transcription factor might specifically regulate the expression of the genes involved in cutin biosynthesis. Therefore, the genetic manipulation of *CD2* gene is promising for the improvement of tomato fruit quality.
The **CD3 gene**: The CD3 contains the conserved domains of fatty acid omega-hydroxylase and exhibits 70% amino acid sequence identity to CYP86A8 (LCR) and 68% to CYP86A4 of Arabidopsis (Fig. 2.13). This

![Sequence Alignment](image.png)

**Figure 2.13** The alignment of CD3 sequence with an Arabidopsis homolog. Score = 830 bits (2143), Expect = 0.0, Method: Compositional matrix adjust. Identities = 392/542 (72%), Positives = 461/542 (85%). CYP86A8 has alkane 1-meroxygenase activity, involved in ω-hydroxylation of fatty acids.
suggests that the CD3 protein probably has alkane 1-monooxygenase activity
and catalyzes the $\omega$-hydroxylation of fatty acids. The CD3 is likely an ER
protein, as predicted by Predotar v. 1.03 (http://urgi.versailles.inra.fr/predotar/
predotar.html) and P2SL (Atalay and Cetin-Atalay 2005; http://www.i-
cancer.org/p2sl/).

A schematic diagram summarizing and integrating the predicted
functions and subcellular localizations of three cutin deficient proteins in the
cutin biosynthesis pathway is shown in Figure 2.14.
Figure 2.14 Model of the putative functions of three cutin deficient genes in the cutin biosynthesis pathway. Predictions: CD1 catalyzes cutin polymerization via successive transesterification of 2-MAG to the growing cutin polymer at the cutin synthesis site. CD2 is a transcription factor in the HD-Zip IV subfamily, possibly regulating the expression of the genes that encode lipid transfer proteins or ABC transporters. CD3 has alkane 1-monoxygenase activity and can catalyze ω-hydroxylation of fatty acids, probably in ER.
REFERENCES


CHAPTER THREE

Physiological and Developmental Characterization of the hcr1 Mutant

3.1 Introduction

Fruit cuticle cracking, also called ‘swell cracking’, ‘shrink cracking’, ‘crazing’ and ‘cuticle blotch’, is a common problem encountered by commercial tomato growers (Dorais 2004). Cuticle cracking downgrades fruit quality as it causes poor appearance such as roughened skin or corky tissues and also reduces fruit shelf life. In the case of severe cracking, such as concentric and radial fruit cracking where large, deep cracks occur on the cuticle, the cracked fruits become essentially unmarketable for fresh consumption, resulting in reduced commercial yield.

A number of physiological and biophysical studies have demonstrated that the development of cuticle cracking is associated with fruit characteristics such as fruit anatomy, shape and growth rate, water status and sugar content (Emmons and Scott, 1998; Dorais 2004). Cracking is also intimately associated with features of the fruit epidermis, such as cuticle thickness, ultrastructure and mechanical properties (Matas et al., 2004, 2005; Lopez-
Casado et al., 2007; Dominguez et al., 2011; Hetzroni et al., 2011). The cuticle biomechanical parameters change during plant growth and are affected by components of fruit cuticular membrane, such as cutin, wax and polysaccharides. In addition, tomato fruit cuticle cracking is considerably influenced by cultivation practices such as growth season and irrigation management, and environmental factors such as air temperature, humidity and illumination (Dorais 2004; Matas et al., 2005; Hahn, 2011). Cuticular cracking initiation typically occurs after fruit absolute growth rates reach maximum values and so growth rate, at the moment the cracks are first observed, is usually unrelated to the actual incidence of cracking (Ehret et al., 1993).

Susceptibility to fruit cuticle cracking has been intensely studied among various tomato cultivars (Emmons and Scott, 1998; Dorais 2004; Matas et al., 2004), leading to the conclusion that many genes may be involved in the development of fruit cracking, and each type of cracking may be influenced by specific genes (Cuartero et al., 1981). However, studies targeting the genetic or molecular mechanisms underlying fruit cracking are extremely limited compared with equivalent morphological and physiological studies, in which has limited the breeding and development of cracking-resistant cultivars (Moctezuma et al., 2003).

To shed new light on the cracking phenomenon and particularly to investigate the underlying molecular bases, a search was performed online of
the ‘Genes that Make Tomatoes’ collection (http://zamir.sgn.cornell.edu/mutants/; Menda et al., 2004) for mutants that were annotated as exhibiting a fruit cracking phenotype. One mutant (# e4702m1) was identified as having substantial fruit cuticle cracking and was named hypercracking 1 (hcr1). In this chapter, the characterization of the physiological and developmental phenotypes of the hcr1 mutant is described and the possible underlying mechanisms are discussed.

### 3.2 Material and Methods

#### 3.2.1 Plant materials

Seeds of hcr1 mutant were obtained from the ‘Genes that Make Tomatoes’ EMS mutagenesis collection (Menda et al., 2004). Tomato plants were grown in the Guterman greenhouse at Cornell University, Ithaca, New York, under 16 hour of light and 8 hour of dark, using standard practices. Due to the low self-pollination rate of hcr1 under greenhouse condition, the flowers of hcr1 and M82 were hand-pollinated and tagged. Fruits from mutant and wild-type plants were harvested at the appropriate number of days after pollination (DAP). For the mature green (MG) stage, fruits were collected when they reached full size, but were still green and the red ripe (RR) stage corresponded to fruits 4-5 days after the color break.
3.2.2 Microscopy

For structural analyses of the fruit pericarp, fruit tissue was fixed and embedded in paraffin as described by Jackson (1992) and 6 μm paraffin sections of fruit pericarp were prepared. Sudan IV, Toluidine Blue and Neutral Red staining were conducted as described in Buda et al. (2009). For estimating cell size and the number of pericarp cell layers, paraffin sections were stained with Toluidine Blue and 10 or more sections were counted, as described in Vrebalov et al. (2009). The size of the parenchyma cells was estimated by counting cell numbers within a certain area using ImageJ software (http://rsbweb.nih.gov/ij/) and 8 or more sections were measured each sample.

3.2.3 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was performed as described previously by Isaacson et al. (2009) with minor modifications. Briefly, whole fruits at young stages (3-9 DAP) were frozen in liquid nitrogen, freeze dried, mounted on aluminum stubs and sputter coated with gold: palladium 60:40 using a Denton Desk II coater. Microscopic observations of fruit surface were made with a Leica 440 scanning electron microscope using an accelerating voltage of 5 kV (Leica, http://www.leica.com).
3.2.4 Statistics

The measurement results of some phenotypes in the chapter 3 and 4 were represented as means ± S.D. (Standard Deviation). The significant differences were determined by the Student’s t-test.

3.3 Results

3.3.1 The phenotypes of the hcr1 mutant

Greenhouse grown hcr1 plants are phenotypically normal during vegetative growth. After emergence of the first inflorescence, necrotic spots gradually developed on some of the newly emerging leaves (Fig. 3.1A) and some necrosis was apparent on the surface of some of the anthers (Fig. 3.1B).

![Image A](image1.png) ![Image B](image2.png)

**Figure 3.1** Necrosis on leaves and anthers of hcr1 mutant
(A) Necrotic spots on a leaf of the hcr1 mutant. Scale bar = 2 mm. (B) Enzymatic discoloration on anther surface of hcr1 mutant. The picture in the bottom right corner shows the normal anther surface of M82. Scale bars = 500 µm.
However, the major phenotypes were apparent on the *hcr1* fruits during their rapid expansion phase, including fruit discoloration (Fig. 3.2), reduced fruit expansion (Fig. 3.3), fruit cracking (Fig. 3.4) and suberization (Fig. 3.5).

**Figure 3.2** Discoloration phenotype of young *hcr1* fruits. Necrotic phenotypes of *hcr1* fruits at (A) 3 DAP (highlighted with an arrowhead). Scale bar = 1 mm. (B) 5 DAP. Scale bar = 1 mm. (C) 9 DAP. Scale bar = 500 µm

**Figure 3.3** The fruit size phenotype of *hcr1*. Comparison of fruit sizes between *hcr1* and M82 at (A) 7 DAP and (B) 14 DAP. Scale bars = 1 cm
Fruit discoloration occurred as early as 3 DAP, and all fruits exhibited some discoloration at 6 DAP (Fig. 3.6). The first visible cracks were generally

Figure 3.4 Fruit cracking in hcr1 fruits. (A) Scanning Electron Micrograph (SEM) of hcr1 fruit surface at 6 DAP showing the small cracks. Scale bar = 50 µm. (B) and (C) cracks on hcr1 fruit at 9 DAP. Scale bars = 500 µm

Figure 3.5 The suberization phenotype of hcr1 fruits, showing different patterns of suberin scars. Scale bars = 1 cm
seen 3-5 days after the appearance of discoloration. However, the most striking phenotype of hcr1 fruit was the deposition of large suberized scars (Fig. 3.5) which typically occurred coincident with the cracks indicating that the suberization is a wounding response to the cracking. Interestingly, parthenocarpic fruits, which typically occur at a low frequency in tomato, showed no surface suberin scars or cracking in the case of hcr1 (Fig. 3.7). From the cross sections of fruits at 14 DAP (Fig. 3.8), it was apparent that the pericarp of hcr1 fruit is far thinner and there are very small locular cavities, or sometimes no apparent cavity compared to that of the M82 wild type fruit.

Figure 3.6  Time course of the occurrence of discoloration or visible cracks on the hcr1 fruit surface.
The morphology of the hcr1 fruit pericarp cells was obviously altered by the mutation, in comparison with that of the wild-type. At 7 DAP, several layers of misshapen cells with aberrant cell wall thickening were observed near the epidermis in hcr1 (Fig. 3.9B), in contrast to well-organized epidermal

Figure 3.7 Parthenocarpic fruits of the hcr1 showing the absence of suberization on the surface. Scale bars = 1 cm

Figure 3.8 Comparisons of fruit cross sections of hcr1 and M82 fruit. Fruit cross sections of hcr1 and M82 at 14 DAP. Scale bars = 1 cm.
cells of M82 (Fig 3.9A). At 14 DAP, the cutin layer of hcr1 fruit (Fig. 3.9D) was significantly thinner than that of M82 (Fig. 3.9C), suggesting a defect in cuticle development. Substantial suberization was apparent associated with the cracked areas of hcr1 fruit (Fig. 3.9F).

Figure 3.9  Light microscopy images of hcr1 fruit surface tissues using a range of stains to highlight different polymer matrices (A-B) Fruit pericarp cross sections of M82 (A) and hcr1 (B) at 7 DAP, stained with Sudan IV, showing disordered epidermal cells with thickened cell walls in hcr1. (C-D) Fruit pericarp cross sections of M82 (C) and hcr1 (D) at 14 DAP, stained with Sudan IV, showing the developing cutin layer in M82 (C), but almost no cutin layer in hcr1 (D). (E-F) Fluorescence images of fruit pericarp cross sections at 21DAP, stained with neutral red, showing a well-developed cutin layer in M82 (E) and the suberized tissues in hcr1(F). (Scale bars = 50 µm)
The average size of hcr1 pericarp parenchyma cells in cross section was substantially less than that of wild-type (Fig. 3.10), indicating an inhibition of the cell expansion in hcr1 fruits.

![Figure 3.10](image)

**Figure 3.10  Cell size comparison of the pericarp of hcr1 and M82 fruit.**  Light microscopy images of pericarp cross section from M82 (A) and hcr1 (B) fruit at 14 DAP in the same magnification, showing inhibition of cell expansion in hcr1(B). Scale bars = 100 µm

### 3.3.2 Abnormal cell, tissue and organ development of hcr1 fruit is associated with aberrant cell expansion and division

To quantify some of the clear anatomical abnormalities of the hcr1 fruit, measurements were made of the pericarp thickness and the ratios of the fresh weights of pericarp versus those of the inner tissues at an early fruit developmental stage, compared with M82 (Fig. 3.11). No differences were detected in pericarp (ovary wall) thickness at 0 DAP (Fig. 3.11A) but significant differences (P-value = 2.8E-06) were present by 3 DAP. The pericarp
thickness of *hcr1* fruits was less than 30% of that of the wild-type pericarp by 9 DAP. The lack of normal pericarp development was also reflected in measurements of the fresh weight ratio of pericarp versus inner tissue in *hcr1* fruit, which was substantially smaller than that of M82 at 3 DAP (Fig. 3.11B). The ratio in *hcr1* fruits continued to decline until 9 DAP in contrast to that in M82, which continued to increase throughout the time course. These results confirmed that the growth of pericarp of *hcr1* fruits was significantly inhibited compared with wild-type fruits.
To determine whether this was a consequence of defective cell expansion or division, the number of pericarp cell layers was counted and the parenchyma cell sizes were measured by light microscopy analysis of pericarp sections (Fig. 3.12). No differences were seen in the number of cell layers in the ovary wall between the \textit{hcr1} mutant and M82 at 0 DAP, but a significant (p-value = 1.4E-05) reduction was present in \textit{hcr1} by 3 DAP. A substantial difference (approximately 30% reduction) from 6 DAP onwards (Fig. 3.12A), indicating that the cell \textit{hcr1} mutation caused a disruption of cell division. For the average areas of pericarp parenchyma cells, no difference was observed between mutant and wild type at 0 and 3 DAP, but by 12 DAP, the average

![Figure 3.12](image)

**Figure 3.12** Inhibition of cell division and expansion in the \textit{hcr1} fruit pericarp. (A) Comparison of the number of fruit pericarp cell layers of \textit{hcr1} and M82. (B) The average areas of pericarp parenchyma cells in \textit{hcr1} and M82 fruit.
area of an *hcr1* parenchyma cell was less than 30% that of M82 at 12 DAP (Fig. 3.12B), indicating a severe inhibition of cell expansion.

### 3.4 Discussion

The *hcr1* mutant exhibits an array of phenotypes that are most evident in fruits. These traits emerge in a distinct chronological order, suggesting that some have causal relationships with others. Essentially no differences were apparent, based on visual inspection and light microscopy between ovaries of *hcr1* and wild-type prior to pollination at 0 DAP. However, by 3 DAP, several traits, including pericarp thickness (Fig. 3.11A), weight ratio of pericarp to inner tissue (Fig. 3.11B) and the number of pericarp cell layers (Fig. 3.12A) showed significant differences between the mutant and wild type. In addition, brown discoloration of the surface of some fruits, indicative of necrosis, was noticeable by 3 DAP (Fig. 3.2A). By 6 DAP, the average size of the parenchyma cells of the *hcr1* pericarp was significantly smaller than that of the control (Fig. 3.12B) and discoloration was prevalent in most fruits, together with visible cracking on the surface of some fruits (Fig. 3.6). By this developmental time point, disordered epidermal cells with thickened wall cells were observed in pericarp cross sections (Fig. 3.9B). Suberization of the areas around the cracks was also commonly observed (Fig. 3.5), presumably representing a wound healing response to fruit cracking.
Other than the severe cracking, the most striking phenotype of the hcr1 fruit is the absence of a fully developed pericarp, which reflects a major impairment in both pericarp cell expansion and cell division. Interestingly, no major phenotypes were observed in other parts of the plant in this regard and the only other plant phenotype was the appearance of mild necrosis on some leaves. A reasonable hypothesis is that the hcr1 mutation impairs cell division and expansion specifically in the pericarp and so as the fruits expand, the biomechanical stresses imposed on the outer pericarp tissues result in rupture and cracking. The cracking is first apparent as microfissures, detectable here only with SEM, which are associated with the early discoloration and necrosis, and these later develop into the major cracks, triggering tissue suberization.

In the next chapter the cloning of the hcr1 gene is described and a model is presented linking hcr1 function with the phenotypes described above.
REFERENCES


CHAPTER FOUR

Cloning and Molecular Characterization of the hcr1 Mutant

4.1 Introduction

The development of fruit epidermis has significant impacts on the horticultural quality of fruits, because fruit epidermis performs vital protection functions, which include minimizing water loss, protecting against pathogen, maintaining fruit integrity and preventing fruit from other physical damages. Fruit cracking and catfacing (corky scars on fruit surface) are common physiological disorder related with fruits epidermis damage. Those disorders are persistent and widespread problem, degrading fruit appearance and quality and subsequently causing serious economic loss. Hypercracking 1 (hcr1), a mutant derived from a tomato line M82 by EMS mutagenesis, show fruit cracking at an early stage and consequent excessive suberization throughout the fruit development. In Chapter Three, we have characterized the phenotypic features and discussed the possible causal relations of some phenotypes. In this chapter, the molecular characterization of the hcr1 gene is described, together with the possible mechanisms underlying the inhibition of
cell expansion/division in hcr1 fruit and a model about hcr1 fruit cracking is presented.

4.2 Material and Methods

4.2.1 Genetic mapping of HCR1

An F$_2$ mapping population was generated by crossing hcr1 mutant with the wild tomato species S. pimpinellifolium (accession LA1589), and then harvesting F$_2$ self-pollinated seeds from F$_1$ progenies. Genomic DNA was extracted from young leaves of seedlings according to the method described by Fulton et al. (1995). Ninety-four plants of the hcr1 F$_2$ population were phenotyped (using fruit cracking as the identifying trait), and then genotyped with 51 genetic markers that are distributed across the 12 chromosomes with an interval about 10~30cM. Genotype and phenotype data were analyzed by Qgene 4.0.

4.2.2 Plasmid constructions and plant transformation

A binary vector pCambia1305.1 was used as the basis of the β-glucuronidase (GUS) reporter (Jefferson et al., 1987) and complementation constructs. All PCRs were performed by Bio-Rad iProof™ High-Fidelity PCR kit. For the native promoter complementation construction, a 3.1kb DNA
fragment located upstream of *HCR1* gene was amplified from M82 genomic DNA using gene specific primers (Forward: 5’-CTTTAGTCTCAGGGCACA TGAA-3’ and Reverse: 5’-TTCTAGCGG ATGAGCATTTGCAC-3’). The native promoter was cloned into the *Pst I* and *BstE II* sites of pCAMBIA1305.1 vector. The coding region of *HCR1* was amplified from cDNA of M82 using gene specific primers (Forward: 5’-CAGTCGTTAAGCCAATGAGTGAATGA TGCTGCACG-3’ and Reverse: 5’- CAGTCGTTGTTACCCCAATGAGTGAATGAT GCTGCACG-3’); and then was subcloned into the vector described above at *BstE II* site. For the 35S promoter construct, the coding DNA of *HCR1* gene was amplified using gene specific primers (Forward: 5’-ACTGCagatctCATGGTGGAAGAAAAATGGTGTGTGGTGAC-3’ and Reverse: 5’-ACCTCAcacgtgGGATGTCTCCCATCAATGCTAGACCA-3’); and then was cloned into the pCAMBIA 1305.1 vector at *Bgl II* and *Pml I* site. For the GUS reporter expression construct, the native promoter was amplified using the gene specific primers (Forward: 5’-CCTTTAGTCTCAGGGCACATGAA-3’ and Reverse: 5’-ACGCCAGccatggTGCCAAGAGATTGAGATTCTTCAAGG-3’), then was cloned into the pCAMBIA 1305.1 vector by replacing CaMV35S promoter fragment using *Pst I* and *Nco I* for the enzyme digestion. The two complementation constructs were transformed into *hcr1* mutant plants by *Agrobacterium*-mediated transformation (Van Eck et al., 2006) using plant transformation facility in UC Davis. The GUS construct was transformed into M82 plants by the *Agrobacterium*-mediated method (Van Eck et al., 2006;
http://irc.igd.cornell.edu/Protocols/Tomatotransform.html) in the plant transformation facility at the Boyce Thompson Institute or Plant Research, Cornell University.

### 4.2.3 Sterol profiling

Tomato leaf tissue (without petioles) was harvested from the leaves at the third node counting from top of each plant when flower buds in the first inflorescence were still small. Fruit pericarp samples were collected from 3 different stages: 15 DAP, MG and RR. Samples were flash frozen in liquid nitrogen and lyophilized in FreeZone Freeze Dry System (Labconco, Model: 77520) for 24 hours and then stored in plastic screw-cap tubes at -80°C until being subjected to lipid extraction.

Extraction was performed as described by Wewer et al. (2011) with minor modifications. A total of 1g of material was used for lipid extraction with 15 ml of chloroform/methanol (2:1). For each extract, a phase separation was effected by addition of 5 ml of 0.145 M NaCl solution. The sample was vortexed and centrifuged for 2 min at 5000 g. The non-polar fraction containing free sterols was removed with a pipette. Two additional extraction of chloroform/methanol (2:1) were performed and the organic phases combined, and the solvent was evaporated under N₂ gas. The polar fraction containing non-free sterols were separated by TLC in acetone:toluene:water
and the lipids isolated from the silica plates with chloroform:methanol (2:1). This fraction was hydrolyzed in 1 ml 1 N methanolic HCl at 90°C for 30 min. After the addition of 1 ml of 0.154 M NaCl solution, the hydrolyzed sterols were extracted with 1 ml of hexane. Sterols were derivatized with 100 μl N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) and analyzed by comparison with authentic standards using GC-MS (GC: 6890 series, HP and Mass Selective detector: Agilent, Model: 5973).

4.2.4 RNA isolation, cDNA synthesis and Quantitative PCR analysis

Pericarp tissue from three biological replicates for each stage were harvested, flash frozen in liquid nitrogen then stored at –80°C. For unpollinated ovaries/fruits (at 0 DAP), whole fruits were sampled as it was difficult to separate ovary wall/pericarp from adjacent tissues. Total RNA was isolated from frozen tissue (10~80 mg) using an RNeasy mini kit (Qiagen) and on-column DNase digestion was performed with the RNase-free DNase set (Qiagen), following the manufacturer’s instructions. A total of 3 μg of total RNA was used for cDNA synthesis with SuperScript III reverse transcriptase and oligo (dT) primers (Invitrogen), according to the manufacturer’s instructions.

Quantitative PCR experiments were performed using 7900HT Real-Time PCR System (Applied Biosystems) with 384-well block module. Twenty microliter qPCR mixtures, prepared with 10 μl 2× HotStart-IT SYBR Green
qPCR Master Mix (Affymetrix, Santa Clara, CA, USA), 6 μl 30-fold diluted cDNA and 4 μl other reaction components (Primers: 0.5 μl each, ROX passive reference dye: 0.4 μl and water: 2.6 μl) were loaded into each well of a 384-well plate. To estimate PCR amplification efficiency, calibration dilution experiments were performed in the same plate using a dilution series (10-fold dilution from $10^x$ to $10^5 \times$ as well as a blank and a $30\times$ dilution) from the cDNA mixture which was mixed with the same volume of cDNA from each sample. The default thermal cycling condition for standard 384-well plate was applied for all qPCR runs (95°C for 10 min, 40 circles: 95°C for 15 seconds, 60°C for 60 seconds), and a dsDNA high-resolution melt curve analysis was followed.

The sequences of oligonucleotide primers are listed in Table 4.1. Specificity of the products was determined by product sequencing, gel electrophoresis and dissociation/melting analysis. qPCR data were analyzed using SDS2.1 software (Applied Biosystems). A mean normalized expression of genes was calculated using Q-Gene software (http://www.gene-quantification.de/download.html) in Microsoft Excel. The RPL2 gene (Yeats et al., 2010) was served as a constitutive control in all gene expression experiments.

### 4.2.5 Protein sequence alignment

ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used for protein sequence alignment.
### Table 4.1  PCR primers used for gene expression analysis

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
<th>PCR length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPL2 (SGN-U581377)</td>
<td>CAGCGGATGTCGTGCTATGAT</td>
<td>GGGATGCTCCACTGGATTCA</td>
<td>154</td>
</tr>
<tr>
<td>3β-HSD/D1 (HCR1-1) (SGN-U582999)</td>
<td>AGCGTACTTTGGATCATTTCCACC</td>
<td>TCCCATAATGCTAGACCA</td>
<td>140</td>
</tr>
<tr>
<td>3β-HSD/D1 (HCR1-2) (SGN-U582999)</td>
<td>GCAAGTGACCAACTAGGATGACCT</td>
<td>ATCTTTCCCAGCCCGTGTTC</td>
<td>108</td>
</tr>
<tr>
<td>3β-HSD/D2 (HCR2) (SGN-U583000)</td>
<td>GGAATCCCAGGTGCTTTAC</td>
<td>CCCAGAAATATCGAAGTCCTCC</td>
<td>117</td>
</tr>
<tr>
<td>Polyphenol Oxidase F (SGN-U577900)</td>
<td>AGCCTTACGTTGATGTTGCT</td>
<td>AGGCAGTAACGGACTTGTC</td>
<td>124</td>
</tr>
<tr>
<td>Polyphenol Oxidase E (SGN-U579941)</td>
<td>GTCTGAGATTTAGTGAGGCT</td>
<td>AGCATTAACCAACACTAGCAAGTC</td>
<td>130</td>
</tr>
<tr>
<td>GDSL-motif lipase/hydrolase (SGN-U585129)</td>
<td>GTAGCATGTTGGACAAGGACC</td>
<td>TTTGCCCTCTCAGATGGATGGAAC</td>
<td>122</td>
</tr>
<tr>
<td>SICER6 (SGN-U566767)</td>
<td>CCAGTGTTCATCCAGAGATTGT</td>
<td>GTCTGAGATGTCACAGTT</td>
<td>171</td>
</tr>
<tr>
<td>Feruloyl-Coenzyme A Transferase (SGN-U562949)</td>
<td>ACGGATTCGGGTTGGGAGA</td>
<td>CTTCATAGCTGAAGCTGGGAGTCC</td>
<td>138</td>
</tr>
<tr>
<td>SICESA1 (SGN-U585892)</td>
<td>AGAGGTATGTGGTGGACTGTG</td>
<td>CCCCTTGGCTCTCCTGGTCTG</td>
<td>99</td>
</tr>
<tr>
<td>SICESA6-like (SGN-U589487)</td>
<td>CTGGGCTGTCTTCTTGGCATCC</td>
<td>ACCACATGGACCTTTGCAAGCA</td>
<td>99</td>
</tr>
<tr>
<td>SICESA3 (SGN-U585891)</td>
<td>GGACCAGATGTCAGGTTGTG</td>
<td>TCACATGGATGACCTTTGCA</td>
<td>95</td>
</tr>
</tbody>
</table>

#### 4.2.6 GUS histochemical staining

Pericarp sections were cut by hand from fresh tissues, and fixed in cooled 80% acetone for about 30 minutes. GUS histochemical staining was performed according to Jefferson et al. (1987).
4.2.7 Subcellular localization of HCR1–tdTOMATO

The protocol used for subcellular localization of HCR1 gene was performed as described in Kelley et al. (2010). The cytoplasmic GFP and ER-GFP markers (which were kindly provided by J.Y. Lee, Delaware Biotechnology Institute, University of Delaware, Newark, DE) and pART-27 vector (Gleave, 1992) are also same as those described in the paper. Briefly, the coding region of HCR1 was amplified from cDNA of M82 using gene specific primers (Forward: 5’-CCGATGctcgagATGGGTGAAGAAAAATGGTGTGTTGGT-3’ and Reverse: 5’-CGGATCggtaccATTCTTCTTGCTTTTGTCAAATATCAAG-3’); and then was subcloned into the pART-27 vector with tdTOMATO (red fluorescence) (Shu et al., 2006) reporter sequence at Xho I and Kpn I site to generate the protein expression structure: 35S::HCR1: tdTOMATO: OCS-term. Confocal microscopy imaging of the co-expression of HCR1-tdTOM and cytoplasmic GFP or ER-GFP marker was observed by transient protein expression in onion epidermal cells.

4.2.8 Cellulose analysis

The procedure for crude cell wall isolation and acid hydrolysis of cell wall was performed as described by Foster et al. (2010). The amount of glucose hydrolyzed from crystalline cellulose was measured using the colorimetric PAHBAH (p-hydroxybenzoic acid hydrazide) assay (Blakeney and
Mutton, 1980), in which the absorbance values were measured by Multiskan EX Microplate Photometer (Thermo Electron Corporation) with 405nm filter.

4.3 Results

4.3.1 The mapping result of HCR1 gene

The *HCR1* gene was first mapped to the middle portion of tomato chromosome two with a substantial QTL (LOD=32) between the markers 1g11430 (2.070) and 2g18030 (2.083) (Fig. 4.1). Following a screen of approximately 6,000 F2 individuals for recombination events by the two markers, the gene was further narrowed down to a 42 kb interval between two flanking CAPS markers: Seq19600 and UDP4F within a BAC contig (corresponding to C02.40_contig20 in version 2.4 of tomato BAC contigs database, http://solgenomics.net) (Fig. 4.2A). A point mutation was found by comparing the genomic DNA sequences of *hcr1* and M82 in this region (Fig. 4.2B). This nucleotide substitution (G→A) is located in the predicted 3’ splicing site of the seventh exon of the *HCR1* gene, which results in the removal of the seventh exon from the mRNA (Fig 4.2B), as confirmed subsequently by the RT-PCR (Fig 4.3). The chromosomal position of *HCR1* is: SL2.40ch02:40139750…40146827.
Figure 4.1  QTL map in the hcr1 mapping population
(A) QTL analysis using a Simple Interval Mapping (SIM)  (B) QTL analysis using Composite Interval Mapping (CIM-LS) with cofactor maker (with blue background) selection. The red line indicates the value of log-likelihood ratio (LOD) and the green line indicates the LOD threshold (>3). The number in parentheses indicates the marker location in cM.
**Figure 4.2  Mapping of the hcr1 locus.** Schematic diagram showing the mapping of hcr1 to chromosome 2 between the marker Seq19600 and UDP4F. (B) HCR1 gene model. The nucleotide substitution G to A at the 3’ splice site results in the removal of the seventh exon from the mRNA of the 3β-HSD/D1 gene, leading to the truncation of the last 142 amino acids from the protein.
4.3.2 Complementation test of the $HCR1$ gene

To validate the identity of the $HCR1$ gene, complementation tests were performed using two plasmid constructs: (i) CaMV35S:: $HCR1$ cDNA and (ii) Native Promoter:: $HCR1$ cDNA. All defective phenotypes of $hcr1$, including fruit discoloration, early cracking and cuticle suberization were entirely absent in the transgenic plants ($T_0$) with either of the complementation constructs (Fig. 4.4). Segregation analysis in the subsequent generation ($T_1$) revealed that the progenies of selected transgenic ($T_0$) lines showed approximately 3:1 segregation ratios. Thus it was confirmed that the single nucleotide mutation in the splicing site of the seventh exon of the $HCR1$ gene was responsible for the $hcr1$ phenotypes.

Figure 4.3 RT-PCR of the $HCR1$ gene

![RT-PCR of the HCR1 gene](image)
The HCR1 gene is predicted to encode a 419 amino acid polypeptide that bears a conserved 3β-HSD multi-domain pfam01073 (3-283aa, 1.27e^-74). A BLAST search against the Arabidopsis protein database showed that the tomato HCR1 protein exhibits a sequence identity of 78% and a similarity of 88% with 3β-Hydroxysteroid Dehydrogenases/C-4 Decarboxylases (At3β-HSD/D1) (Fig. 4.5). The HCR1 contains a conserved glycine and aspartic residues, TGGXGXXAX_{18}D near the N terminus, which is required to form the coenzyme-binding site (Rahier et al., 2006). The highly conserved YX₃K segment [156~160aa], assigned to the catalytic center, is also present in HCR1 (Jornvall et al., 1995, Rahier, 2011). A transmembrane helix
Figure 4.5 Sequence alignment of HCR1 and At3β-HSD/D1.
CLUSTAL 2.1 multiple sequence alignment, Score = 67.0
Identities = 285/384 (74%), Positives=325/384 (85%)

[379-413aa] close to the C-terminal was predicted by TMHMM 2.0
(http://www.dtu.dk/services/TMHMM/) and a putative ER retention and
retrieving signal KSKKN motif of type-I ER membrane protein was found at the
C-terminus (http://elm.eu.org/). In the hcr1 mutant, the removal of the
seventh exon from the HCR1 mRNA leads to the truncation of the last 142
amino acids (from 278aa to 419aa) from the protein (Fig. 4.2B), which would
almost certainly result in the loss of activity and correct subcellular localization.
A homolog of the HCR1 gene, named here HCR2 (SGN-U583000), was identified in the tomato genome sequence by a BLAST search. Both HCR1 and HCR2 are located on the chromosome 2, but with a distance of about 21 Mbp. The predicted HCR2 protein has 562 amino acids and shows and a sequence identity to the HCR1 protein of 84% (Fig. 4.6).

Figure 4.6  Sequence alignment of HCR1 and HCR2
CLUSTAL 2.1 multiple sequence alignment  Score = 78.0
Identities = 310/371 (84%), Positives = 338/371 (91%)
The enzymatic characteristics of two 3β-HSD/D isoenzymes in *Arabidopsis* have been characterized (Rahier et al., 2006; 2009; Rahier, 2011). At3β-HSD/Ds are bifunctional short-chain proteins with 3β-hydroxysteroid-dehydrogenase/C4-decarboxylase activities, acting as a key enzyme for C-4 demethylation in the sterol biosynthesis pathway. HCR1, HCR2 and the two *Arabidopsis* At3β-HSD/Ds show high amino acid sequence identity (more than 65%) and structural similarity to each other, suggesting that HCR1 and HCR2 most likely have 3β-hydroxysteroid-dehydrogenase/C4-decarboxylase activities.

### 4.3.4 *hcr1* mutation causes a mild reduction of sterol contents in *hcr1* fruits

Since the *HCR1* gene encodes a key enzyme in sterol pathway, the contents of bulk sterols in leaves and fruit pericarp at three developmental stages were measured by GC-MS (Fig. 4.7 and Table 4.1). Despite the loss of HCR1 enzymatic activity, the sterol profiles of *hcr1* leaves were similar to those of M82 during vegetative growth of plants. Strikingly, the conjugated sterols such as sterol glucoside (SG) and acylated sterol glucoside (ASG) were dominant in tomato leaves, accounting for more than 98% of total sterols (Table 4.1), which contrasts with the content of conjugated sterols in *Arabidopsis*: only account for about 20% of total leaf sterols (Wewer, 2011).
The total sterol content of the *hcr1* pericarp was reduced to 84~89% of wild-type levels at 15 DAP, mature green and red ripe stages, which while relatively small, was statistically significant in all three stages (Fig. 4.7, Table

![Graph showing sterol profile of fruit pericarps and leaves in M82 and hcr1.](image)

**Figure 4.7** The sterol profile of fruit pericarps and leaves in M82 and *hcr1*. Total free sterols and total steryl glucosides are shown on the left and the right, respectively. (Mean ± SD of N = 3. **P < 0.01 by two-tailed t-test.**
4.1. Similarly, the conjugated sterols (particularly sitosterol and stigmasterol) were dominant in tomato fruit pericarp in all three stages and accounted for more than 90% of total sterols (Table 4.1), which was consistent with previous findings (Whitaker et al., 1988, 1991 and 2008).

4.3.5 Spatial and developmental expression patterns of HCR1 gene

The spatial and developmental expression pattern of the *HCR1* gene was determined using the GUS reporter driven by the *HCR1* gene promoter. The results indicated that *HCR1* gene is ubiquitously expressed in all tomato tissues (Fig. 4.8, 4.9 and 4.10). GUS accumulation was relatively low in fruits at 1 DAP (Fig. 4.8 A), but quickly peaked by 3~5 DAP (Fig. 4.8B). Expression was more sparse in older fruits, first showing absence in some parenchyma cells at 12 DAP (Fig. 4.8C) and then in mature green (Fig. 4.8D) and red ripe (Fig. 4.8E) fruits, only appearing in the epidermis, sporadically in some cells near the epidermis and in vascular tissues.

In stems, GUS staining was detected in all cells and was particularly abundant in young tissues (Fig. 4.9A), then gradually declined as the tissues aged (Fig. 4.9B, C), until staining was no longer detectable in well-developed stem tissues (Fig. 4.9D). The *HCR1* gene expression in petioles showed a similar developmental pattern to that in stems (Fig. 4.9E-H). Interestingly, GUS staining of rings of endodermal cells and xylem rays (Fig. 4.9C) was observed in the stem. From the results of the GUS reporter experiments, it
Table 4.1  Sterol composition of steryl lipids from leaves and pericarp tissues at three stages in M82 and hcr1 mutant (µg/g DW)

<table>
<thead>
<tr>
<th>Sterols</th>
<th>15 DAP Pericarp</th>
<th>Mature Green Pericarp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M82</td>
<td>hcr1</td>
</tr>
<tr>
<td><strong>Free sterols</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campesterol</td>
<td>3.3 ± 0.5</td>
<td>3.0 ± 1.4</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>12.2 ± 3.8</td>
<td>13.4 ± 3.6</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>42.7 ± 7.1</td>
<td>46.5 ± 8.0</td>
</tr>
<tr>
<td>Cycloartenol</td>
<td>1.3 ± 0.3</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>5.1 ± 0.8</td>
<td>6.0 ± 0.7</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>64.7 ± 11.3</td>
<td>70.5 ± 13.4</td>
</tr>
<tr>
<td><strong>SG+ASG</strong> *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campesterol</td>
<td>17.9 ± 5.3</td>
<td>17.3 ± 2.6</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td><strong>119.0 ± 3.0</strong></td>
<td><strong>97.8 ± 6.6</strong></td>
</tr>
<tr>
<td>Sitosterol</td>
<td><strong>637.7 ± 24.5</strong></td>
<td><strong>543.3 ± 26.4</strong></td>
</tr>
<tr>
<td>Cycloartenol</td>
<td>10.3 ± 0.8</td>
<td>9.7 ± 0.8</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>26.6 ± 3.7</td>
<td>29.4 ± 5.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>811.6 ± 15.9</strong></td>
<td><strong>697.4 ± 29.8</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sterols</th>
<th>Red Ripe Pericarp</th>
<th>Leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M82</td>
<td>hcr1</td>
</tr>
<tr>
<td><strong>Free sterols</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campesterol</td>
<td>6.0 ± 1.6</td>
<td>5.7 ± 0.8</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td><strong>27.4 ± 2.5</strong></td>
<td><strong>21.7 ± 1.3</strong></td>
</tr>
<tr>
<td>Sitosterol</td>
<td><strong>26.6 ± 2.0</strong></td>
<td><strong>20.6 ± 0.5</strong></td>
</tr>
<tr>
<td>Cycloartenol</td>
<td>1.0 ± 0.5</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.3 ± 0.1</td>
<td>1.5 ± 0.8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>62.3 ± 4.7</strong></td>
<td><strong>50.9 ± 1.1</strong></td>
</tr>
<tr>
<td><strong>SG+ASG</strong> *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campesterol</td>
<td>28.7 ± 2.3</td>
<td>24.5 ± 0.9</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td><strong>294.5 ± 14.7</strong></td>
<td><strong>227.0 ± 5.1</strong></td>
</tr>
<tr>
<td>Sitosterol</td>
<td><strong>714.5 ± 20.8</strong></td>
<td><strong>671.6 ± 24.2</strong></td>
</tr>
<tr>
<td>Cycloartenol</td>
<td><strong>12.8 ± 0.9</strong></td>
<td><strong>17.3 ± 1.7</strong></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>11.0 ± 1.1</td>
<td>11.1 ± 1.3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1061 ± 33.3</strong></td>
<td><strong>951.5 ± 20.3</strong></td>
</tr>
</tbody>
</table>

* SG = Steryl glycosides; ASG = Acylated steryl glycosides
** Data represent the mean (µg/g dry weight) of three biological replicates ± SE. Major differences are highlighted in bold font.
was concluded that the *HCR1* gene is abundantly expressed in the regions undergoing rapid cell division and expansion.

**Figure 4.8  Spatial and developmental expression analysis of *HCR1* gene in fruit pericarp using the GUS reporter system.** GUS staining of fruit pericarp at different stages: 1 DAP (A), 4 DAP (B), 12 DAP (C), mature green (MG) (D) and red ripe (RR) (E). Scale bars (A,B) = 100 µm, (C, D, E) = 500 µm.
Figure 4.9  Spatial and developmental expression analysis of HCR1 gene in stems and petioles using the GUS reporter system. (A-D) A developmental stages of tomato stems from younger (A) to older (D). (E-H) Petioles at different stages from younger (E) to older (H). Scale bars (D, E) = 100 µm, Scale bars of others = 500 µm.
Developmental expression analysis of the \textit{HCR1} and \textit{HCR2} genes was also carried out by quantitative PCR. Two sets of \textit{HCR1} specific primers were designed (Fig. 4.11A): the hcr1-1 primers, matching two specific region on the ninth and tenth exon (spanning the ninth intron), were used to detect all \textit{HCR1} cDNA species and the hcr1-2 primers, matching two specific regions on seventh and eighth exon (spanning the seventh intron), were used to detect the presence or absence of the deleted seventh exon of the \textit{hcr1} gene. The qPCR result showed that the \textit{hcr1} transcripts in \textit{hcr1} plant were reduced to only about 10\% of that of M82 at 3~9 DAP (Fig 4.11B); however, low levels of wild type \textit{HCR1} transcripts with the seventh exon region intact were detected,

\textbf{Figure 4.10} \textit{HCR1} gene expression analysis in other tomato organs and tissues using the GUS reporter system. GUS staining in anther (A), ovule (B), sepal (C) and root (D). Scale bars = 100 µm.
Figure 4.11 Developmental expression analyses of HCR1 transcripts in fruit pericarp by qPCR. (A) The primer design schematic for HCR1 gene expression analysis. (B) HCR1 gene expression was determined using the hcr1-1 primer set, which is near to the 3'UTR region of the cDNA to detect all cDNA species. (C) HCR1 gene expression using the hcr1-2 primer set, which is designed to detect the presence of the deleted seventh exon in cDNA of hcr1 mutant.
albeit only at 0.5~1% of wild-type levels (Fig 4.11C). A possible explanation for this result is that most hcr1 mRNAs (about 90%) without the 7th exon were eliminated by cellular quality control machineries due to the instability of the defective mRNA, but that very small amounts (0.5~1%) of functional HCR1 mRNA accumulated in the hcr1 mutant because of fault-tolerant splicing during mRNA transcription. It is noteworthy that the gene expression pattern in M82 fruit probed by the two sets of PCR primers showed a similar pattern: HCR1 gene expression levels peaked at 3 DAP then declined gradually during fruit development and were reduced to about 20% of peak values at 12 DAP. This result is consistent with the expression pattern of the HCR1 gene observed by the GUS reporter assay.

The HCR2 gene displayed relatively consistent expression profiles across all time-points, but expression was slightly reduced in the hcr1 mutant (Fig. 4.12).

4.3.6 Several metabolism processes related to fruit development were affected by the hcr1 mutation.

The wide range phenotypic traits shown by hcr1, including fruit discoloration, suberin deposition, abnormal cuticle development and cell division and expansion, suggested that many metabolism pathways were altered by the hcr1 mutation. To confirm this, the expression patterns of several marker genes related to these processes were investigated.
Polyphenol oxidases (PPOs) catalyze the $O_2$-dependent oxidation of phenolics to quinones. The secondary reactions of quinones lead to the formation of polymeric brown or black pigments (Thipyapong et al., 1997, 2007). The previous study showed that mechanical wounding and pathogen infection can induce the expression of the $PPO$-F gene in tomato, suggesting that this gene may involve in the observed brown discoloration of the hcr1 fruit. 

qPCR analysis of $PPO$-F expression (Fig. 4.13A) indicated that un-pollinated
tomato fruits have the most abundant \textit{PPO-F} transcripts and that after pollination, \textit{PPO-F} transcripts in M82 fruits decreased dramatically and by 6 DAP were reduced to only 1~4 \% of the original levels at 0 DAP. In contrast, \textit{PPO-F} transcript levels in \textit{hcr1} fruit remained high during this period and were about 60-fold greater than that of the wild-type, which is consistent with \textit{PPO-F} contributing to the fruit discoloration in \textit{hcr1}.

\textbf{Figure 4.13}  \textbf{The expression patterns of marker genes related to fruit discoloration and suberization by qPCR.}  \textbf{The expression of (A)} \textit{Polyphenol Oxidase F} and (B) \textit{Feruloyl-Coenzyme A Transferase} by qPCR was used as a measure of phenolic compound formation and suberin biosynthesis, respectively, during M82 and \textit{hcr1} fruit development.
Feruloyl-CoA transferase (AT5G41040) is essential for the incorporation of ferulate into suberin and has been identified as a key enzyme required for suberin synthesis in *Arabidopsis* (Molina et al., 2009). The expression pattern of the closest homolog of AT5G41040 in tomato (SGN-U562949, Solyc03g097500.2) was analyzed to gain insights into the suberin biosynthetic pathway during fruit development of M82 and *hcr1* fruit (Fig. 4.13B). qPCR results showed that this gene (SGN-U562949) was consistently expressed at a very low level in M82 plants, but was dramatically up-regulated in *hcr1* 6 days after pollination in comparison with the wild-type. These expression patterns are consistent with the observation that suberin synthesis lagged behind fruit discoloration and the first appearance of cracks.

Two marker genes which are associated with cutin and wax biosynthesis were selected for monitoring the cuticle formation of *hcr1* fruit. *CD1* catalyzes the polymerization of cutin in situ via successive trans-esterification of 2-MHG to the growing cutin polymers (see chapter 2).

*LeCer6* encodes an essential enzyme of a very-long-chain fatty acid (VLCFA) elongase complex, which is required for aliphatic wax biosynthesis (Vogg et al., 2004; Leide et al., 2007) and so its expression would be expected to correlate with wax deposition. The qPCR results revealed that the expression patterns of *CD1* and *LeCer6* in M82 were quite similar with both genes were maximally expressed during the rapid expansion phase of fruits at 12 DAP or later (Fig. 4.14). In contrast, the expression of these two genes in
*hcr1* fruit substantially lagged behind that of M82, suggesting that, as observed microscopically, cuticle development was apparently delayed in the *hcr1* mutant.

![Figure 4.14](image)

**Figure 4.14** The expression patterns of marker genes related to fruit cuticle biosynthesis. The expression of (A) the cutin polymerase *CD1* gene and (B) the wax biosynthesis *CER6* gene during M82 and *hcr1* fruit development was quantified by qPCR.

#### 4.3.7 *hcr1* mutant exhibit reduced levels of cellulose in fruit pericarp

Several studies have suggested that structural sterols may be involved in cellulose biosynthesis (Peng et al., 2002; Schrick et al., 2004; Bessueille et al., 2009). To examine the potential association between sterols and
cellulose biosynthesis in the hcr1 mutant, the crystalline cellulose content in the pericarp of hcr1 and wild type fruit at 12 DAP and the mature green stage was measured. The results showed that the crystalline cellulose levels in hcr1 pericarp at both stages were reduced to 65% and 70% of that of the wild-type, respectively (Fig. 4.15), suggesting significant cell-wall cellulose deficiency in both stages. This result was consistent with the studies of three other sterol biosynthesis mutants from Arabidopsis, fk, hyd1 and smt1/cph (Schrick et al., 2004).

![Figure 4.15 Crystalline cellulose content of hcr1 and M82 fruit pericarp at 12 DAP and mature green stages.](Image)

(Mean ± SD of N = 4; ** P < 0.01 by two-tailed t-test.)
4.3.8 The gene expression of three subunits of cellulose synthase complex

It was hypothesized that the observed decrease of cellulose levels in cell wall extracts from hcr1 pericarp could have resulted from reduced expression of cellulose synthase. To test this, we assessed the transcript levels of three subunits of cellulose synthase using qPCR (SlCesA1, SlCesA3, and SlCesA2/SICesA6). Through phylogenetic analysis, SlCesA1 and SlCesA3, which appear to be orthologous to Arabidopsis CesA1 and CesA3, respectively, were identified in the tomato genome. But SlCesA6 and SlCesA2 could not be unambiguously distinguished from each other because the similarity of these two genes was too high. The result showed that the difference in expression levels of SlCesA1 and SlCesA3 (Fig. 4.16A, B) between M82 and hcr1 mutant were small, but statistical differences were detected in gene expression level of CesA6-like subunit (Fig. 4.16C). However, considering that this difference was small (0.5~1 fold only) and the function of CesA6-like subunit can be compensated by other CesAs, the function of the cellulose synthase complex for primary cell wall formation may not be substantially affected by hcr1 lesion at the level of transcript abundance.
Figure 4.16  The expression patterns of marker genes related to cellulose biosynthesis. The expression patterns of SlCesA1(A), SlCesA3(B) and SlCesA6-like (C) during M82 and hcr1 fruit development were quantified by qPCR.
4.3.9 Subcellular localization of HCR1 protein

HCR1 protein possesses the putative ER retrieval signal KXXXX-COOH at the C-terminus. In order to verify subcellular localization, confocal imaging analysis was performed of HCR1 fused to the tdTOMATO fluorescent reporter protein following transient expression in onion epidermal cells. The HCR1: tdTOMATO fusion protein co-localized with a GFP–ER marker protein, but not with a cytoplasmic GFP marker (Fig. 4.11). This result confirmed that HCR1 resides in ER membranes, in agreement with the current model that the ER is the site of sterol synthesis (Carland, et al., 2010).

4.4 Discussion

4.4.1 A function for 3β-hydroxysteroid-dehydrogenase/C4-decarboxylase in the plant sterol biosynthetic pathway.

Map based cloning revealed that the HCR1 gene encodes a predicted 3β-hydroxysteroid-dehydrogenase/C4-decarboxylase, a bifunctional enzyme that catalyzes the demethylation of two C-4 methyl groups in the sterol biosynthesis pathway (Rehier et al., 2006). This pathway has been well-defined in plants by biochemical and molecular studies of sterol mutants (Benveniste, 2004; Boutte and Grebe, 2009). Sterols become functional
molecules only after the removal of two methyl groups from the C-4 position of sterol precursors. The metabolic enzymes, catalytic mechanism and the structure of multienzyme complex for sterol C-4 demethylation in higher plants have been thoroughly reviewed (e.g. Rahier, 2011). In brief, three individual enzymes, including a sterol methyl oxidase (SMO) (Lees et al., 1995; Darnet...
and Rahier, 2004), a 3β-hydroxysteroid-dehydrogenase/C4-decarboxylase (3β-HSD/D) (Rahier et al., 2006, 2009) and a 3-ketosteroid reductase (SR), are likely tethered as a membrane-bound multienzyme complex and sequentially participate in each C-4 demethylation step. Two 3β-HSD/D genes were identified from Arabidopsis and their functions have been well-characterized (Rahier et al., 2006, 2009). Substrate screening studies showed that these two isoenzymes require free 3β-hydroxyl and C-4-carboxyl groups for the enzymatic reaction, but are quite tolerant of structural variations in the sterol nucleus and side chains. An enzymatic activity assay revealed that the apparent catalytic rates of At3β-HSD/Ds are very high compared to those of other enzymes in the postsqualene sterol pathway, suggesting they are not rate-limiting steps in the overall sterol biosynthesis.

4.4.2 Sterols and plant growth

Sterols are essential lipid components of cell membranes that provide stability to the phospholipid bilayer and play a critical role in maintaining membrane integrity and function. They may directly or indirectly modulate the activity of integral membrane proteins, including enzymes, ion channels and signal transduction components (Schaller, 2003, 2004; Boutte and Grebe, 2009). Plant sterols are also the precursors of the brassinosteroid (BR) hormones and so have considerable influence on various plant physiological processes (Asami et al., 2005; Wang et al., 2006; Li, 2010). Molecular and
biochemical approaches to characterize sterol mutants with altered sterol profiles, or genetic manipulation of sterol-related genes in Arabidopsis have revealed pleiotropic roles of sterols in plant growth and development. These include patterning of organs (e.g. embryo, vein, shoot and root), cell expansion/elongation, cell polarity and proliferation, reactive oxygen species (ROS) regulation, cellulose biosynthesis, root hair initiation and hormone signaling (Diener et al., 2000; Jang et al., 2000; Schrick et al., 2000, 2004; Carland et al., 2002, 2010; Peng et al., 2002; Souter et al., 2002, 2004; Willemsen et al., 2003; Men et al., 2008; Pose and Botella, 2009; Ovecka et al., 2010). However, the molecular mechanism underlying the specific phenotypes observed in many sterol-deficient mutants remain obscure and molecular downstream targets of sterols are largely unknown.

A common phenotype noted in many sterol synthetic mutants is a dwarfism, which is thought to be caused by an inhibition of cell expansion and division (Schrick et al., 2000, 2004; Pose et al., 2009; Carland et al., 2010). Brassinosteroids (BRs), derived from campesterol, are involved in numerous plant developmental processes, such as cell expansion and elongation, cell division and cell wall regeneration (Clouse and Sasse 1998; Cano-Delgado et al., 2004; Nemhauser et al., 2004). However, the dwarfed phenotype of most sterol biosynthetic mutants cannot be rescued by exogenous BR treatment, indicating that structural sterol molecules are also crucial for cell expansion and division (Fujioka and Yokota, 2003; Schaller, 2003; Carland et al., 2010).
Gene expression analysis demonstrated that exogenous application of both typical (sitosterol and stigmasterol) and atypical (e.g. 8, 14-diene sterols) sterols can activate the expression of genes involved in cell expansion and division, while sterol biosynthetic genes are regulated by several growth-promoting hormones, including BRs and auxin (He et al., 2003). This suggests the existence of a signaling crosstalk among sterols, hormones and the genes involved cell growth; however, knowledge of the regulatory role of sterols in transcriptional regulation of cell growth-related genes is still extremely limited.

Plant cell expansion and division require coordinated biosynthesis and restructuring of the cell wall, the major load bearing component of which is the cellulose/xyloglucan network (Rose and Bennett, 1999). Various studies have indicated that sterols are involved in cellulose biosynthesis, which in turn suggests that perturbations in sterol biosynthesis may adversely affect cell wall formation and lead to impaired cell division and expansion. Peng et al. (2002) reported that a CesA glucosyltransferase may initiate glucan polymerization using sitosterol-β-glucoside (SG) as a primer (Peng et al., 2002). This proposed model, however, could not adequately explain the causal relations between sterol-deficiency and cellulose deficiency in some sterol mutants, as the primer role of SG may be easily fulfilled by residual levels of steryl glycosides (DeBolt et al., 2009). In a comparative study of three sterol mutants, it was found that the *fackel*, *hydra1* and *smt1/cph*
mutants shared similar cellular ultrastructural features, such as incomplete cell walls, aberrant wall thickenings and ectopic callose or lignin deposits, despite distinct variances in their sterol profiles. In addition, all three mutants exhibited a cellulose deficiency in the seedling cell walls, but normal levels of pectins and sugars, leading the authors to speculate that sterols are involved in cellulose synthesis (Schrick et al., 2004).

In recent years, the structure, composition, and possible functions of membrane raft-like domains of the plant PM have been studied (Peskan et al., 2000; Bessueille et al., 2009; Mongrand et al., 2010; Carmona-Salazar et al., 2011). Sterols and sphingolipids are enriched in these raft-like plasma membrane domains and the self-associating properties of these two types of lipids are considered to be the main driving force for the formation of stable membrane domains (Silvius, 2005). It was reported that detergent-resistant membranes (DRMs) isolated from the PM of hybrid aspen contain key wall polysaccharide synthases, including callose synthase and cellulose synthases (Bessueille et al., 2009). Indeed, more than 70% of the total glucan synthase activities in the PM were associated with this sterol-enriched DRM fraction (Bessueille et al., 2009). These results further support the possible involvement of structural sterols in cellulose biosynthesis. In addition, proteomic analyses have indicated that a high proportion of other proteins, such as ABCB19, OsRac1 and REMORIN protein, are associated with the DRM fraction, prompting speculation that DRMs may be involved in various
physiological processes such as signaling, immunity and environmental stress response etc. (Morel et al., 2006; Fujiwara, et al., 2009; Raffaele et al., 2009; Titapiwatanakun et al., 2009). Although detergent-resistant membranes may not explicitly define functional membrane rafts in plants (Boutte and Grebe, 2009; Tanner et al., 2011), such research has considerably increased our knowledge of the role of sterols in plant growth and development.

Several recent studies have demonstrated that the growth-suppressed phenotypes of two sterol synthetic mutants may be associated with the altered ROS production (Pose et al., 2009a, 2009b; Kim et al., 2010). ROS are important in the regulation of many biological processes, such as stress responses and systemic signaling and abnormal ROS accumulation can trigger oxidative stress and inhibition of cell growth, further inducing programmed cell death in relatively severe cases (Gill and Tuteja, 2010; Torres, 2010). The sqe1-5/dry2 Arabidopsis mutant shows pleiotropic developmental defects including a dwarfed appearance and pale green leaves, and was identified as a sterol synthetic mutant with a point mutation in the Squalene Epoxidase 1 (SQE1) gene. Notably, the strong developmental defects were not correlated with changes in sterol composition, but rather with the altered production of ROS. In addition, this mutation caused the mislocalization of the RHD2 NADPH oxidase and induced ectopic accumulation of ROS in root hair cells, leading to the suggestion that sterols play an essential role in ROS regulation (Pose et al., 2009a, 2009b). In
another study, the Arabidopsis cyp51a2 mutant, which displays pleiotropic defects, such as stunted hypocotyls, short roots, reduced cell elongation and seedling lethality (Kim et al., 2005) was shown to have a defect in a gene encoding an obtusifoliol 14α-demethylase enzyme, which performs the 14α-demethylation step in the sterol biosynthetic pathway. Transcriptome and proteome profiling analyses suggested that changes in membrane sterol composition in the cyp51a2 mutant trigger the generation of ROS and ethylene, and eventually cause seedling senescence and death (Kim et al., 2010). Other possible roles of sterols in cell expansion and division have also been proposed. For example, the cell expansion defects of the smt mutants may be related to the regulatory roles of sterols in cell cycling (Carland et al., 2010).

It is apparent that sterols are involved in many and diverse aspects of plant cell function, through multiple complex mechanisms. This is reflected in the presence of a broad array of major sterols, numerous intermediates and a range of structural modifications (e.g. glycosylation and acylation), perturbations of which have been identified in different sterol mutants (Chaturvedi et al., 2011; Schrick et al., 2011; Wewer et al., 2011). Considering the pleiotropic phenotypes, the presence of many sterol molecular species and diverse molecular downstream targets of different sterols, the functional characterization of plant sterol remains challenging.
4.4.3 The sterol profile of *hcr1* fruit may be partially complemented by *HCR2*

It is common that redundancy among enzymes or even pathways ensures a balanced sterol composition and maintains sterols at adequate levels in plants (Carland et al., 2010). This homeostasis mechanism underscores the crucial role of sterols on plant growth and development. In *Arabidopsis*, two 3β-HSD/D isoenzymes can catalyze the demethylation without obvious preference *in vitro* between substrates with one or two substituents at C-4 position, suggesting that the enzymes have enzymes complementary characteristics (Rahier, 2011). Similarly, in this study, the two tomato 3β-HSD/D isoenzymes (*HCR1* and *HCR2*) share a high level of sequence identity (84%) (Fig. 4.5), suggesting that they may functionally compensate for each other. Most *hcr1* gene transcripts (>99%) were defective, but the *hcr1* mutant plants were phenotypically normal and the sterol profiles of the *hcr1* leaves were unaffected. A possible explanation for this result is that the function of *HCR1* gene is complemented by *HCR2*.

Redundancy between *HCR1* and *HCR2* is also consistent with relatively small differences in fruit sterol composition between *hcr1* and M82: only an 11-16% reduction in total sterols was detected in *hcr1* pericarp samples at three developmental stages. The expression pattern analysis of the two genes provides further support for such redundancy as *HCR1* was expressed
at low levels at 0 DAP, but quickly peaked at 3 DAP, and then gradually reduced to about of 20% of the peak level at 12 DAP (Fig. 4.9). In contrast, *HCR2* was expressed at low levels with a relatively stable expression pattern (Fig. 4.10) during early fruit development. A comparison of the relative transcript levels of the two genes showed that the ratio of normalized expression values of *HCR1* to *HCR2* was about 6-fold at 3DAP, but almost zero at 12DAP. Transcript abundance analysis using an RNA-Seq digital expression database of the Ailsa Craig tomato variety showed that the number of normalized reads of *HCR1* was about two-fold higher than that of *HCR2* at 10 DAP (Matas et al., 2011). This result is in agreement with the assessment of relative transcript abundance of the two genes based on qPCR.

**4.4.4 Evidence that sterols influence cellulose synthesis in *hcr1* fruit**

Cellulose is the dominant structural component of plant cell walls. However, despite considerable progress made over the last decade, many fundamental questions related to the mechanisms of cellulose synthesis and our knowledge of its regulation and modulation is very limited (Mutwil et al., 2008; Endler and Persson, 2011; Guerriero et al., 2010).

As mentioned above, studies of sterol synthetic mutants (Schrick et al., 2004) and biochemical association of cellulose synthase activity with sterol rich PM extracts (Bessueille et al., 2009) provide indirect evidence that sterols are important for cellulose synthesis. The crystalline cellulose content in the
pericarp of *hcr1* fruit at 12 DAP or the mature green stage was only about 65-70% of that in wild-type, which is a similar reduction to that reported for the three sterol-deficient *Arabidopsis* mutants (Schrick, et al., 2004). This suggests a possible causal relationship between sterol deficiency in the *hcr1* fruit and the reduction in crystalline cellulose, which could in turn lead to an inhibition of cell division/expansion, impaired pericarp development and fruit cracking. Unlike *fk, hyd1* and *smt1/cph* mutants, the levels of bulk sterols in the pericarp of *hcr1* mutant is only 11-16% less than those of wild-type, while the reduction of crystalline cellulose content in the pericarp of *hcr1* fruit is substantial. However, redundancy of function between HCR1 and HCR2 provides a possible explanation for this discrepancy.

### 4.4.5 Is the *hcr1* growth inhibition phenotype associated with other physiological disorders?

Rapid and transient generation of ROS is considered to be an important component of the resistance response of plants to various abiotic and biotic stresses (Neill et al., 2002). ROS can serve as direct protective agents through their toxicity, but may also regulate many physiological processes such as stress responses, systemic signaling and programed cell death (Gill and Tuteja, 2010; Torres, 2010). ROS generation in plant cells is normally counterbalanced by the action of antioxidant enzymes or other redox molecules. However, abiotic or biotic stresses may lead to the overproduction
of ROS, which are highly reactive and toxic to proteins, lipids and DNA, resulting in oxidative stress. Recently, studies of two sterol synthetic mutants, dry2/sqe1-5 and cyp51A2, revealed that the reduction of the sterol biosynthetic enzyme activities can trigger the ectopic accumulation of ROS and eventually cause abnormal phenotypes (Pose et al., 2009b; Kim et al., 2010), although the underlying molecular mechanism remain unclear.

In hcr1 mutant, some phenotypic traits, including leaf necrosis and fruit discoloration are similar to several physiological stresses (e.g. programed cell death) triggered by ROS accumulation. In an experiment involving 3βHSD/D gene silencing using virus induced gene silencing (VIGS), 4-carboxysterol, an atypical sterol derivative which account for 2-3% of the total sterol content was detected in infected Nicotiana benthamiana plants (Rahier et al., 2006, 2011). Hence we cannot rule out the possibility that some of the hcr1 phenotypes are associated with ROS accumulation or abnormal sterols due to the reduced enzymatic activities of 3βHSD/D.

4.4.6 Proposed model of hcr1 mutation

Based on the information gathered from morphological, cytological, biochemical and molecular experiments, a model is proposed to explain how the HCR1 gene lesion causes the observed fruit phenotypes (Figure 4.18.) In this model, a nucleotide substitution (G to A) at the 3’ splice site of the HCR1 gene leads to the loss of the enzymatic activity of 3β-HSD/D, which consequently results in the sterol deficiency of hcr1 fruit, especially during the
Figure 4.18 Proposed model for the relations between phenotypes and *hcr1* mutation
high expression of HCR1 gene at 3 to 9 DAP. The sterol deficiency further causes reduced cellulose synthesis in the fruit pericarp cells walls, resulting in inhibited the cell division and expansion. The mechanically weak pericarp cracks during early fruit expansion and the observed fruit suberization is a normal wound-healing response to this cracking.
REFERENCES


membranes (DRMs) associated with OsRac1-mediated innate immunity in rice. *Plant Cell Physiol.*, **50**(7), 1191-1200.


CHAPTER 5

Conclusions and Future Directions

5.1 Conclusions

As described in the introduction, the structure, composition and other biological features of fruit cuticle have significant effects on many economically important traits, such as storability/shelf life, surface appearance and resistance to desiccation, microbial infection or cracking. Tomato is an ideal model species for fruit cuticle biology study, due to its economic importance, typical cuticle composition and the ease of cuticle isolation. Chapter two describes the mapping of three genes related to fruit cutin biosynthesis in this model system. The $CD1$ gene encodes a GDSL-motif lipase/hydrolase, which catalyzes extracellular cutin polymerization at the cuticle formation site. The $CD2$ gene encodes a putative transcription factor in the IV group of the homeodomain-leucine zipper family (HD-Zip IV). The $CD3$ gene was identified as a homolog of Arabidopsis CYP86A, which catalyzes the $\omega$-hydroxylation to C16 and C18 fatty acids that act a precursors for cutin formation.

In chapters three and four, the research focuses on the detailed characterization of the $hypercracking \ 1 \ (hcr1)$ mutant. The $HCR1$ gene encodes a $3\beta$-hydroxysteroid dehydrogenase/C-4 decarboxylase ($3\beta$-HSD/D1),
which is a key enzyme in the sterol biosynthesis pathway. The hcr1 lesion results in a reduction in sterol levels in the hcr1 fruit pericarp. Phenotypic and biochemical analysis of hcr1 suggests that early fruit cracking is probably caused by severe inhibition of cell division and expansion and possibly defective polysaccharide cell wall biosynthesis, suggesting a role for sterols in cellulose synthesis. The observed extensive fruit suberization is likely a wound-healing response to early fruit cracking.

At least 50 genes are thought to be involved in cuticle biosynthesis, monomer transportation and regulation in Arabidopsis. In tomato, however, knowledge of fruit cuticle biology is less advanced. Although on the characterization of the four cuticle and fruit surface mutants presented here represents a significant contribution to the field, more effort is needed to gain a better understanding of the mechanism underlying biosynthesis, metabolism, assembly and regulation of tomato fruit cuticle.

5.2 Future Directions

5.2.1 Further study of the three cutin-deficient mutants

We have gained valuable information about the function of the CD1 gene. However, a future challenge is to demonstrate the cutin polymerization activity of CD1 protein with the presumed native substrate, 2MHG, in-vitro.
CD2 is a promising candidate gene for developing new plant varieties with desired fruit traits via genetic manipulation. Knowledge about the function of the CD2 gene is still limited, and it has yet to be shown to encode a bona fide transcription factor. If this is in fact the case, the target genes of CD2 should be identified, together with the molecular pathways involved in regulating cutin and cuticle related gene expression. With respect to the CD3 gene, the functions of its homologs in Arabidopsis have been studied in some detail (Wellesen et al., 2001; Rupasinghe et al., 2007). However, it may be useful to validate the fatty acid hydroxylase activity of CD3 protein. The cd3 mutant shows a dramatic cutin reduction in fruit cutin but no visible phenotypes in other organs. Therefore, genetic manipulation of the CD3 gene is also a promising strategy for improving fruit quality.

5.2.2 Outline of future studies of the hcr1 mutant

The studies of the hcr1 mutant described here represent significant progress towards understanding the genetic and molecular mechanism underlying the cuticle cracking in the hcr1 fruits. Briefly, the early fruit cracking of hcr1 likely results from the developmentally abnormal and structurally weak pericarp as a consequence of the inhibition of cell expansion and division. The molecular link between sterol deficiency and this reduced cell expansions and division has still to be established; however, several sets of data, including some generated in the analysis of hcr1 fruit, point to a connection between
abnormal sterol profiles and defective cellulose synthesis, which can be
directly related to cell wall architecture and cell expansion or division.

As mentioned before, studies of the functional role of sterols in plants
are still challenging, due to their pleiotropic nature and the diversity of sterol
species. Currently, the following approaches might be valuable in order to
explore possible mechanisms underlying the cell growth inhibition in hcr1 fruit.

**More accurate sterol profiling:** In the sterol profiling experiment,
only the total sterol content was measured. The transient substrate of the
3βHSD/D enzyme, 4-carboxysterol is a compound with a steroid-like structure.
Unanswered questions include whether 4-carboxysterol or other abnormal
sterols accumulate in hcr1 fruit and whether such abnormal sterols are
toxic to plant cells. In addition, with the development of new analytical technologies,
the profile of sterol lipids can be more accurately quantified by Q-TOF mass
spectrometry methods (Wewer et al., 2011). If the sterol content of M82 and
hcr1 were compared at an early fruit stage (e.g. at 3 DAP), it might provide
new insights into the sterol deficiency in hcr1 fruits at this critical stage.

**RNA-Seq analysis:** An array of distinct phenotypes was observed in
hcr1 fruits during the rapid expansion phase. Consequently, various
physiological processes were likely perturbed in a specific *relative
chronological order*. RNA-Seq is a valuable tool for transcriptomics and could
be used to monitor these physiological changes at the transcriptional level. It
is possible that the downstream targets affected by the hcr1 mutation could be
identified through the differential expression analysis of genes at an early fruit growth stage (e.g. at 0-6 DAP).

5.2.3 Future directions for the study of fruit cuticle biology in tomato

Forward and reverse genetic study of fruit cuticle-related genes:

More than 50 genes involved in cuticle biosynthesis have been identified in *Arabidopsis*, maize or rice. Identifying the counterparts of those cuticle-related genes in the tomato genome via comparative analysis would lay the groundwork for genetic manipulation and for a better understanding of fruit cuticle biosynthesis. Some candidate genes in tomato may be identified by comparing sequence identities with cloned cuticle genes. For some members in a large gene family, phylogenetic analysis may provide important clues for candidate gene prediction. The confirmation of the function of candidate genes in tomato can be carried out by gene targeted mutagenesis approaches, such as RNAi, artificial miRNAs and gene overexpression.

Several tomato mutant collections based on EMS or fast neutron mutagenesis approaches have been released by different research labs. This information is accessible online and seeds of these mutants are available upon request to the tomato scientific community (http://zamir.sgn.cornell.edu/mutants/; http://tgrc.ucdavis.edu/; http://tomatoma.nbrp.jp/index.jsp). These mutant collections provide valuable genetic material for fruit cuticle biology study. For those mutants caused by the mutation of unknown genes, or with
important/interesting phenotypic traits, map-based cloning is still an effective approach to identify the gene functions, especially in the post-genome era.

In addition, some high-throughput techniques such as “TILLING” and “FOX hunting” are powerful tools for fruit cuticle studies. Targeting Induced Local Lesions In Genomes (TILLING) platforms represent a high-throughput reverse genetic strategy for screening mutations of targeted genes. TILLING technology can discover a series of allelic mutations that are very useful for genetic analysis. With the progress of next-generation sequencing technology, detection of induced mutations in several TILLING populations (e.g. rice and wheat) have been performed on the Illumina platform (Tsai et al., 2011). The application of TILLING in tomato is still in its early stages. Rigola et al. (2009) described a KeyPoint approach to identify the mutants of the tomato eIF4E gene based on screening more than 3000 M\textsubscript{2} plants in a single 454 (GS FLX) sequencing run (Rigola et al., 2009). The project “TILLING resources for the tomato functional genomics community” is making substantial progress in the UCD Genome Center TILLING Laboratory (http://tilling.ucdavis.edu/index.php/Tomato_Tilling).

Another mutagenesis screen strategy, “FOX hunting” (Full-length cDNA Over-eXpressor gene hunting), can also be used to identify gene functions in plants. This gain-of-function approach is simple and effective, and allows functional analysis of genes whose action may be masked in conventional knockout approaches due to gene redundancy (Nakamura et al.,
2007; Kondou et al., 2009; Higuchi et al., 2011). This approach has another advantage in that the gene can be transferred to many different plants by transformation once gene function is known.

**Genetic manipulation of cuticle related genes for fruit quality improvement:** It has been suggested that the cuticle may affect fruit storability by providing structural support or reducing fruit water loss (Saladié et al., 2007; Kosma et al., 2010). Increased understanding of the molecular mechanisms underlying fruit cuticle biosynthesis would provide an efficient strategy to improve fruit shelf-life or other important quality traits by genetic engineering of cuticle-related genes. For example, the research described here demonstrated that the CD2 putative transcription factor may specifically regulate the expression of some cutin-related genes without interrupting other plant physiological processes. By manipulation of CD2 gene expression via overexpression, RNAi or other genetic approaches, it may be possible to develop new tomato varieties with long shelf-life or other desired fruit traits.
APPENDIX I

Genetic markers for the mapping of the 4 cuticle mutants

(Tomato var. M82/S. pimpinellifolium population)

<table>
<thead>
<tr>
<th>Location on Chr.</th>
<th>Marker Name</th>
<th>Marker Category</th>
<th>Restriction Enzyme</th>
<th>Marker Quality</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0155</td>
<td>5g51970</td>
<td>CAPS</td>
<td>Xba I</td>
<td>**</td>
</tr>
<tr>
<td>1.02</td>
<td>TG184</td>
<td>dCAPS</td>
<td>Ssp I</td>
<td>*</td>
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<tr>
<td>1.032</td>
<td>SSR98</td>
<td>SSR</td>
<td></td>
<td>**</td>
</tr>
<tr>
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<td>5g13700</td>
<td>CAPS</td>
<td>Cfo I</td>
<td>**</td>
</tr>
<tr>
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<td>4g02070</td>
<td>Indel</td>
<td></td>
<td>**</td>
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<tr>
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<td>3g08030</td>
<td>CAPS</td>
<td>Hinc II</td>
<td>***</td>
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<td>4g00090</td>
<td>CAPS</td>
<td>Rsa I</td>
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<td>CAPS</td>
<td>Hinf I</td>
<td>***</td>
</tr>
<tr>
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<td>CAPS</td>
<td>Hind III</td>
<td>**</td>
</tr>
<tr>
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<td>tg59</td>
<td>CAPS</td>
<td>BstN I</td>
<td>***</td>
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<td>CAPS</td>
<td>EcoR I</td>
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<td>CAPS</td>
<td>Mse I</td>
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<td>Alu I</td>
<td>**</td>
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<td>CAPS</td>
<td>Msp I</td>
<td>**</td>
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<td>1g10240</td>
<td>CAPS</td>
<td>Dpn II</td>
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<td>CAPS</td>
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<td>Indel</td>
<td></td>
<td>**</td>
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<td>dCAPS</td>
<td>Cfo I (=Hha I)</td>
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Note: "Tomato-EXPEN 2000" map is used for the of marker location of genetic markers.
* OK, but not a good marker  ** A good marker  *** A very good marker
## Table 2.2: Phenotype and Genotyping Data of 4 Cuticle Mutants

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**Genotype:**
- Genotype: 1
Table 2.3  The phenotypic and genotype data for cdl (cm14) gene line mapping
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Table 2.4: The phenotypic and genotyping data for cmt (cmt15) gene mapping
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Table 2.5 The phenotype and genotypic data for c2 (cm12) gene fine mapping.

Note: M1404A M16909 and M16404 three markers are designed according the BAC sequence of C01L00026020.1 (134kb).
### Table 2.6 The phenotype and genotyping data for *cd3* (cm20) gene mapping

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Table 2.7: The phenotype and genotypic data for heart (cmnt7) gene mapping
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Table 2.8: The Phenotype and Genotype data for hcr1 (cm7) gene line mapping.