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How did nature engineer the highest surface lipid accumulation among plants? Exceptional expression of acyl-lipid-associated genes for the assembly of extracellular triacylglycerol by Bayberry (*Myrica pensylvanica*) fruits☆

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ABSTRACT

Bayberry (*Myrica pensylvanica*) fruits are covered with a remarkably thick layer of crystalline wax consisting of triacylglycerol (TAG) and diacylglycerol (DAG) esterified exclusively with saturated fatty acids. As the only plant known to accumulate soluble glycerolipids as a major component of surface waxes, Bayberry represents a novel system to investigate neutral lipid biosynthesis and lipid secretion by vegetative plant cells. The assembly of Bayberry wax is distinct from conventional TAG and other surface waxes, and instead proceeds through a pathway related to cutin synthesis (Simpson and Ohlrogge, 2016). In this study, microscopic examination revealed that the fruit tissue that produces and secretes wax (Bayberry knobs) is fully developed before wax accumulates and that wax is secreted to the surface without cell disruption. Comparison of transcript expression to genetically related tissues (Bayberry leaves, *M. rubra* fruits), cutin-rich tomato and cherry fruit epidermis, and to oil-rich mesocarp and seeds, revealed exceptionally high expression of 13 transcripts for acyl-lipid metabolism together with down-regulation of fatty acid oxidases and desaturases. The predicted protein sequences of the most highly expressed lipid-related enzyme-encoding transcripts in Bayberry knobs are 100% identical to the sequences from Bayberry leaves, which do not produce surface DAG or TAG. Together, these results indicate that TAG biosynthesis and secretion in Bayberry is achieved by both up and down-regulation of a small subset of genes related to the biosynthesis of cutin and saturated fatty acids, and also implies that modifications in gene expression, rather than evolution of new gene functions, was the major mechanism by which Bayberry evolved its specialized lipid metabolism. This article is part of a Special Issue entitled: Plant Lipid Biology edited by Kent D. Chapman and Ivo Feussner.

1. Introduction

The external surface of aerial plant tissues is covered with a hydrophobic barrier called the cuticle. The major constituents of the cuticle are acyl-lipid based and include the insoluble polyester cutin and soluble waxes that are embedded within and on top of cutin. While the hydrophobic cuticle layer is essential for development and survival of all plants, the composition and abundance of cuticular lipids differ between plant species [1,2]. One striking example is the fruit of Bayberry (notably *Myrica* [or *Morella*] *pensylvanica*) which are covered with a thick layer of surface wax that constitutes over 30% of its dry weight, representing the highest reported accumulation of surface wax in plants [3]. In addition, the surface wax is composed primarily of glycerolipids, notably triacylglycerol (TAG) and diacylglycerol (DAG) with saturated fatty acids [3–5]. These are lipids typically found in plant seeds, pollen, and some fruit mesocarps, and no other plant has been reported to contain TAG or DAG as major constituents of surface waxes. Unlike seed oils, Bayberry surface TAGs are not used as an energy/carbon reserve for the plant during germination, but instead the wax may be important to attract birds for seed dispersal [6]. The abundance and physical properties of Bayberry wax also make it a popular source of wax for candle making.

Previously, we used a combination of molecular species analysis, microscopy, and kinetic labeling with [^{14}C]-lipid precursors to demonstrate that Bayberry surface glycerolipids are synthesized by a pathway distinctly different from conventional membrane or storage glycerolipid

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synthesis [3]. While there are still several unknowns regarding mechanisms for Bayberry wax biosynthesis, the key findings that pointed toward a novel pathway for TAG synthesis were: (1) detection of *sn*-2 MAG as an initial intermediate in the biosynthesis of Bayberry wax; (2) analysis of the kinetics of radiolabeled DAG and TAG synthesis, and the distribution of radiolabeled acyl chains on their glycerol backbones indicated distinct acyl donor pools for MAG, DAG, and TAG synthesis and suggested acyl-CoA independent reactions for DAG and TAG synthesis; (3) in vivo and in vitro evidence indicated *sn*-2 MAG may be an acyl donor for DAG and TAG synthesis; (4) the final assembly of TAG occurs extracellularly. The fact that *sn*-2 MAG is also an intermediate in the production of cutin [7] and that *sn*-2 MAG can serve as an acyl donor for extracellular assembly of the cutin polyester [8,9] suggested that the Bayberry surface TAG synthesis pathway may have evolved as an adaptation of cutin synthesis.

In this study, Bayberry's surface lipid biology, fruit development, knob microanatomy and gene expression were examined. We show that the most highly expressed lipid-associated genes in the Bayberry wax producing tissue (knobs) are associated with the production of C16 saturated fatty acids, the assembly of cutin, and secretion of surface lipids. Expression profiles, abundance, and gene sequences were also compared to tissues of other plant species that produce abundant cuticle lipids or triacylglycerols. This comparison demonstrated that Bayberry is exceptional in the expression of a small subset of lipid-related genes and that changes in their protein sequences may not have been required for Bayberry to evolve its unique surface lipid biology. Together, this analysis of Bayberry surface wax production may aid in understanding genes and mechanisms that are available for glycerolipid assembly in non-seed tissue and for glycerolipid secretion from cells and onto external plant surfaces.

2. Materials and methods

2.1. Plant material

Myrica pensylvanica fruits were collected from plants on the campus of Michigan State University (42°43N, 84°23W). Experiments were conducted on tissue harvested from 2011 to 2015. Fruits were used fresh (i.e. within minutes after collection from plants) or were immediately frozen in liquid nitrogen and stored at −80°C for later analysis.

2.2. Wax and lipid extraction and analysis

Surface waxes were extracted from Bayberry fruits by immersing the fruits for up to 30 s in chloroform. Extraction of knob lipids was performed according to [10]. Intact wax was analyzed by high-temperature gas chromatography using a DB5-HT column as described by Simpson and Ohlrogge [3]. Bayberry knob cutin and fatty acid methyl esters from intact lipids were prepared and analyzed according to procedures in [11].

2.3. Microscopy of Bayberry fruits

Standard light microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM) were used to visualize Bayberry knobs. Details for light microscopy and SEM are described in Simpson and Ohlrogge [3]. For TEM, both chemical fixation and high pressure freezing followed by chemical fixation were used. For chemical fixation, Bayberry fruits were placed immediately in fixation buffer containing 4% formaldehyde and 2.5% glyceraldehyde in 0.1 M sodium cacodylate buffer. Samples were post-fixed in 1% osmium tetroxide and then subjected to an ethanol dehydration series. Samples were slowly embedded in 14310 ultra bed resin (Electron Microscopy Sciences) and polymerized at 60 °C for 5 days. High-pressure freezing and freeze substitution weredone on some samples and performed as described in [12]. Ultrathin sections for TEM examination were prepared with a diamond knife on a Power Tome_XL microtome (Brockeler Instruments), and then placed of Formvar-coated copper grids. Post-staining was done with 2% uranyl acetate and Reynolds lead citrate. Samples were viewed under a JEOL 100CX TEM.

2.4. RNA-seq of Bayberry knob tissue

Details for RNA extraction and sequencing for Bayberry knobs and leaves are described in Simpson and Ohlrogge [3]. Gene expression data for *B. napus* [13], oil palm [14], tomato [15], and cherry [16] were obtained from the supplemental files of the respective manuscripts as indicated in Supplemental Dataset 1. RNA-seq data for *M. rubra* fruits [17] was downloaded from NCBI and re-assembled according to the parameters described in Simpson and Ohlrogge [3]. To compare between species, we associated each contig(s) from the respective libraries to its Arabidopsis homolog.

3. Results and discussion

3.1. Comparison of Bayberry wax composition and accumulation to other species and tissues

As shown in Fig. 1, the surfaces of Bayberry fruits are coated with an extremely thick and unusual layer of crystalline wax. At maturity, the wax coverage is 8700 μg cm^{-2} [3] which is 10 to 1000-fold higher than most other plant species, including carnauba leaves, a commercial source of plant wax (Supplemental Figure 1) [2,18]. The composition of Bayberry surface wax also differs from other previously characterized plant surface lipids. Instead Bayberry wax is composed almost exclusively of the glycerolipids triacylglycerol (TAG), diacylglycerol (DAG), and monoacylglycerol (MAG) and the fatty acid composition of these structures consists of unmodified and completely saturated fatty acids (85% palmitate, 14% myristate, 1% stearate). The accumulation of TAG, as opposed to conventional surface lipid structures, makes Bayberry wax chemically similar to oil-seed lipids. However, we previously demonstrated that the pathway for Bayberry glycerolipid synthesis is not related to oil-seed lipids but is to the synthesis of the insoluble surface glycerolipid polyester cutin [3].

The glycerolipids in Bayberry wax accumulate over 8 weeks at a rate of over 700 μg FA cm^{-2} day^{-1} or 0.4 μg FA gFW^{-1} $hour^{-1}$ and at maturity represent approximately 30% of the dry weight of the entire fruit, and 56% of the mass of the tissues that produce and secrete the wax (i.e. knobs). The accumulation of TAG in Bayberry wax is similar to *B. napus* embryos (40–45% oil per dry weight), but less than oil palm mesocarp (90% oil per dry weight). Bayberry wax accumulates to levels that are also 10-fold higher than tomato fruit cutin, and over 100-fold higher compared to Arabidopsis stems [1,19]. Bayberry wax also differs from cutin by accumulating on fully formed tissue [3], while cutin is primarily deposited on young and growing plant tissues [20].

The production of a secreted soluble glycerolipid by Bayberry is somewhat analogous to the glycerolipid estolides that accumulate on the stigmas of some plant species, notably in *Solanaceae*. These structures consist of 18:1 and 18:2-ω-hydroxylated fatty acids that are esterified to two or three positions of the glycerol backbone and in addition form estolide oligomers with an average of 8 acyl chains per glycerol [21]. The biosynthetic pathway for these glycerol polyesters has not been determined; however, in petunia stigmas, a P450 fatty acid hydroxylase, closely related to P450s required for cutin fatty acid production, was identified as important for the synthesis of estolides [22]. One study reported that the estolides can accumulate to 10–20% of the fresh weight of tobacco stigma buds [23]. While both Bayberry fruit and tobacco stigma accumulate abundant surface TAG, unlike Bayberry, the stigma glycerolipids appear to be deposited on the surface via rupture of underlying cells [24], presumably after intracellular assembly. In addition, we could not detect any ω-hydroxylated fatty acids in Bayberry wax glycerolipids.

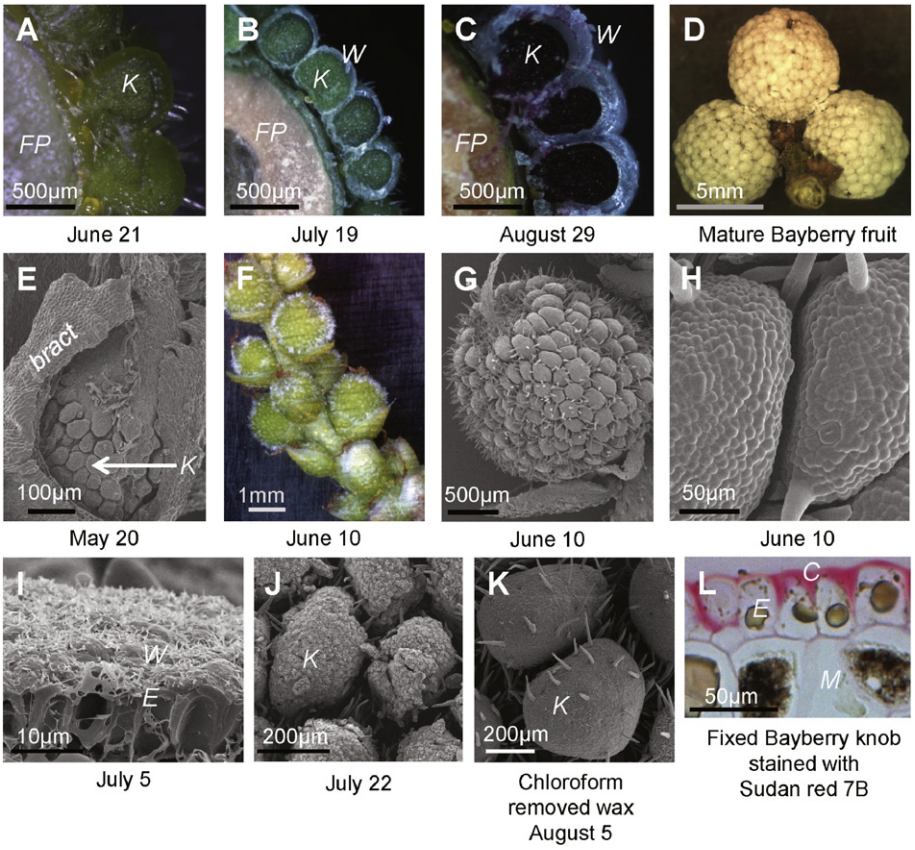

Fig. 1. Wax accumulation on the surfaces of Bayberry fruits/knobs through development. The dates below each image reflect the development of a typical Bayberry fruit in Michigan, USA (42°N, 84°W) during the years 2011–2014. (A–D) Cross-sections of Bayberry fruits. Sections were made on freshly harvested fruits and photographed with a dissecting microscope. Wax was not visible but could be detected by GC in June 21 fruits. (E) SEM image of a dissected flower exposing a developing fruit with knobs. The fruit is within the subtending bracts and the raised tissues on the fruit are the knobs. (F) Light photograph of a developing fruit cluster. (G) SEM image of a whole fruit early in its development. (H) Magnification of the knob surface in G. (I) SEM cross-section of a single knob illustrating wax coverage at less than 10% of final accumulation. (J) SEM of the surface of knobs with wax coverage at ~50% of final accumulation. (K) SEM of the surfaces of knobs after wax was removed by dipping in chloroform. (L) A fixed Bayberry knob stained for lipid with Sudan Red 7b. Surface wax was removed by the chemical fixation of the tissue. Symbols in figures: "K" = knobs; "FP"= fruit proper (i.e. fruit not including the knobs); "W"= wax; "C" = cutin; "M" = knob mesophyll.

3.2. Bayberry fruit development and initiation and secretion of the wax layer

Bayberry fruit deposits its surface wax continuously over approximately 8 weeks, and the surface wax persists on the fruits through the fall and winter months. The wax does not accumulate on the surfaces of the drupe fruit-proper, but instead is produced by and accumulates on unusual multicellular structures (called knobs) that protrude from the fruit surface (Fig. 1). Each ~5 mm (diameter) fruit is completely covered with 200–250 of the ~500 μm (diameter) knobs. We could not find any reports describing the development and morphology of knobs or similar large protrusions from fruit surfaces. To better understand the biology underlying the specialized lipid metabolism of Bayberry, we further characterized aspects of fruit development from pollination to lipid secretion that have not previously been described.

Bayberry fruits were clearly visible throughout the female shrubs 2–3 weeks after pollen production was observed on the male plants. Between 5 and 15 fruits developed per 1–3 cm long branch (Fig. 1F). The knobs were detected very early in fruit development, when the fruit was still inside the flower tissue. Thus, the knobs form concurrently with the fruit-proper, rather than later in development as an outgrowth from more developed tissue (Fig. 1E). By early June, most of the green fruits had expanded past their subtending flower bracts and were fully visible throughout the shrub; however, the glycerolipid surface wax was not detectable at that stage (Fig. 1G, H).

At early stages of wax accumulation, wax crystals were scattered across the surfaces of numerous cells on each single knob (Fig. 1I) and by mid-July knob surfaces were completely covered with wax (Fig. 1J). Significantly, during wax accumulation, we did not observe any fracturing of the knob surfaces. Furthermore, removing the wax later in the season with chloroform revealed an intact knob surface, and fruits stained with Sudan Red 7b revealed a contiguous cutin layer (Fig. 1K, L). These observations, coupled with the prolonged period of wax accumulation (~8 weeks) and inability to detect lipids within the cells by confocal microscopy [3], indicate that the wax is actively secreted from the knobs, rather than released via disintegration of knob cells that have filled with lipids. This process is analogous to cutin/suberin, surface wax, and sporopollenin polymer secretion [25,26]. However, it differs from the cell rupturing observed in stigma estolides [24] and pollen coat lipids [27].

The ultrastructure of the knobs was also examined by light and transmission electron microscopy (TEM). Knob epidermal cells were rectangular with a diameter of 20–30 μm and tightly packed around the circumference of the tissue (Fig. 2A). Internal cells were more spherical in shape, with a diameter of 40–70 μm, and irregularly arranged within the knobs. TEM of the epidermal cells identified chloroplasts, mitochondria, and ER, but no clearly defined lipid storage structures (i.e. lipid droplets). A close association of the ER with the plasma membrane was observed, which may be contact sites associated with lipid secretion [28] (Fig. 2B,C). In contrast, the internal cells were organelle poor and many were filled with electron-dense globules. Similarly, stained globules were evident in some epidermal cells, but none appeared to fill the entire cell (Fig. 2D). The globules likely did not contain lipids, since osmium tetroxide (used in TEM preparation) does not stain

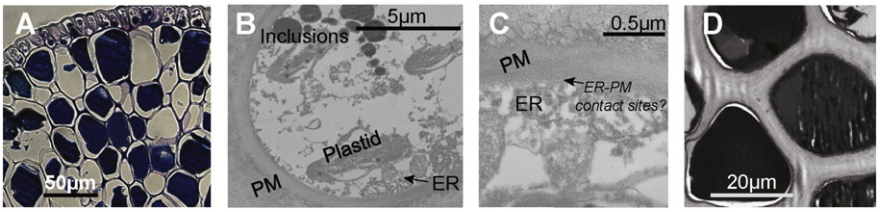

Fig. 2. Ultrastructure of Bayberry knobs. Knobs were harvested in early July and had a total lipid content (including wax) of less than 1000 μg fruit^{-1}. (A) Toluidine-blue-stained section of a knob prior to imaging with TEM. (B) TEM of a knob epidermal cell. (C) Magnification of B showing plasma membrane (PM) and endoplasmic reticulum (ER) region. (D) TEM image of an internal knob cell, not believed to participate in wax synthesis. These cells were filled with highly electron -dense structures that may represent anthocyanins/tannins (see text). Samples shown in panels A–C were chemically fixed, while the sample in panel D was fixed by high pressure freezing (HPF).

saturated lipids. Furthermore, confocal microscopy after staining with neutral lipid dyes [3] indicated that lipid droplets were not present within the knobs. Instead, the electron-dense globules may represent vacuoles filled with phenolic compounds, such as anthocyanins and/or tannins, which are abundant in *Myricaceae* fruits [17] and that are stained by osmium tetroxide through complexes with their O-dihydroxy groups [29].

The subcellular morphology of the epidermal cells, compared to the internal knob cells, also suggested that the epidermis is the only cell layer directly involved in wax production and secretion. This observation was consistent with the fact that staining did not reveal any wax in the interstitial spaces below the epidermis [3]. However, we cannot yet rule out the possibility that some underlying cells may be involved in wax production.

3.3. Features of transcript expression in Bayberry knobs associated with its highly specialized lipid metabolism

RNA-seq was used to identify and quantify gene transcripts that are associated with the very large accumulation of saturated TAG, DAG, and MAG on the surfaces of Bayberry fruits. Knob cDNA was sequenced at seven intervals through development, encompassing a time when the wax layer was undetectable (days 10 and 15), to when wax levels reached 50% of the final yield (day 61) (Supplemental Figure 2). As previously described, 13 of the 55 most highly expressed transcripts in the knobs encode for proteins associated with plant acyl-lipid metabolism [3]. For comparison, in oil crops *B. napus* and oil palm, transcripts for no more than four lipid-related enzymes are represented within their 55 most highly expressed transcripts (Supplemental Table 1). A significant feature of the highly expressed transcripts in Bayberry knobs is that 8 are annotated as biosynthetic enzymes that together can define a pathway from the production of saturated fatty acids to the assembly of Bayberry surface wax.

During wax synthesis, the most highly expressed transcript that is associated with fatty acid synthesis (FAS) is annotated as fatty acid thioesterase B (FATB), the enzyme that releases 16:0 fatty acids from acyl-ACP intermediates of FAS. Five other highly expressed transcripts in Bayberry knobs are annotated as acyltransferases required for assembly of the extracellular glycerolipid cutin. These include two isoforms of *sn*-2 GPATs [30,31]; defective in cuticular ridges (DCR) [32]; and two GDSL-motif lipases/transacylases [8,33]. In contrast, transcripts encoding other glycerolipid acyltransferases, (i.e. Kennedy pathway enzymes) were expressed at levels at least 50-fold lower. The secretion of the wax to the surface may be facilitated by the ABCG transporters and also lipid transfer proteins (LTPs) [25]. Indeed, Bayberry knobs highly express transcripts for one ABCG transporter and three LTPs. Based on homology to Arabidopsis and other plants, these Bayberry proteins are strong candidates to participate in the active secretion of wax to the fruit surfaces.

In the sections below, we describe how very high expression or down-regulation of these and other acyl-lipid genes likely contributed to the evolution of Bayberry's unique wax. Although transcript levels do not directly indicate protein abundance or enzyme activity, transcript expression level and protein amount are generally well correlated for abundant mRNAs [34,35]. We also note that other less abundant and non-lipid-related transcripts that are not considered in this study may be important for the assembly and secretion of Bayberry surface wax.

3.4. Expression of acyl-ACP thioesterases is exceptional in Bayberry knobs

The fatty acid composition of Bayberry surface wax is unique in comparison to other plant surface waxes and also when compared to other soluble glycerolipids in plants. The fatty acids detected in Bayberry surface glycerolipids are all saturated with chain lengths of C16 and C14, with trace amounts of C18. No unsaturated fatty acids or fatty acids with a chain length less than C14 or greater than C18 were detected in the wax at any point though development [3]. Glycerolipids with an entirely saturated long-chain fatty acid composition are not reported for plant membranes or storage lipids [36]. Although some plants, such as *Cuphea*, accumulate greater than 95 mol percent saturated fatty acids in their seed oil, the fatty acids in TAG of these *Cuphea* species are short or medium chain (8:0–12:0) and confer a lower melting point compared to the di- and tri- 16:0 glycerolipids in Bayberry wax [3,36–38].

During the 51-day sampling period of Bayberry knob RNA, the amount of unsaturated fatty acids in the knobs (of a single fruit), which approximates the quantity of polar lipids, was between 15 and 20 μg. During the same period, surface wax on a single fruit increased from undetectable levels to 4000–5000 μg. (Supplemental Figure 3). Most of the unsaturated fatty acids accumulated early in knob development, and the amount of unsaturated fatty acids in the knobs did not change as the surface wax layer grew. Therefore, during times when surface wax was most actively deposited, over 99% of the fatty acids synthesized by knob cells were saturated and used almost exclusively for surface wax synthesis.

How did Bayberry evolve the capacity to specifically synthesize and incorporate only saturated fatty acids into glycerolipids destined for its surface wax? During fatty acid synthesis, the acyl-ACP thioesterase FATB catalyzes the release of 16:0 from acyl-carrier protein (ACP) while the enzyme FATA is most active with 18:1-ACP (formed via 18:0 by stearoyl-ACP desaturase (SAD)) [39,40]. Following the initiation of wax synthesis (days 10–15), transcripts for FATB increased over 5-fold (from 170 to 1000 RPKM) and became the most highly expressed transcript of the FAS pathway and were within the top 40–50 most highly expressed transcripts in the knobs. Furthermore, FATB was expressed 20–40-fold higher than transcripts encoding FATA (Fig. 3A). In contrast to Bayberry, for other plant tissues compared here (tomato and cherry epidermis, *B. napus*, oil palm), FATB is not the highest expressed enzyme of FAS and the ratio of its expression to FATA is between one and five (Fig. 3A). Furthermore, even compared to tomato and cherry epidermis, which produce greater than 85% 16:0 derived fatty acids for cutin, Bayberry is exceptional both in its very high expression of FATB and in the ratio of expression of FATB to FATA transcripts.

Another notable feature of the fatty acid composition of Bayberry wax that may be related to the very high expression of FATB is that

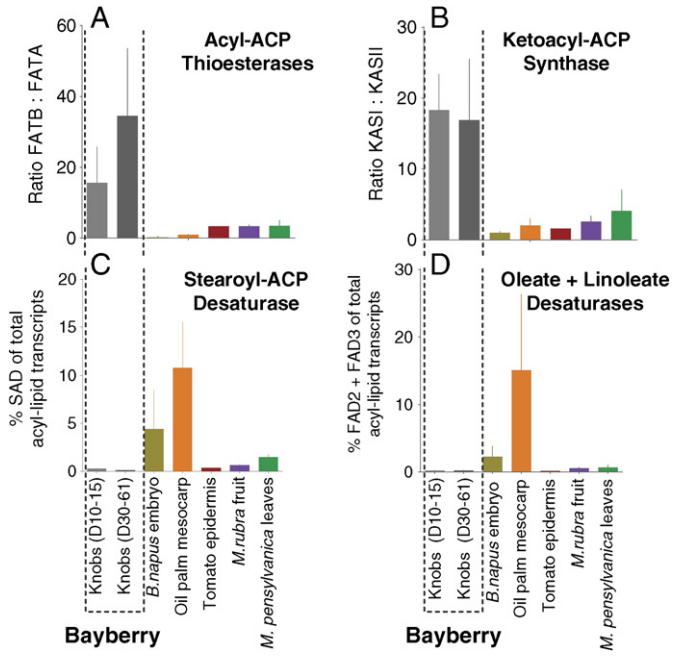

Fig. 3. Specialized expression of genes associated with biosynthesis of greater than 99% saturated fatty acids by Bayberry knobs. For Bayberry knobs, average expression (RPKM) was calculated for stages when wax was not visible (days 10–15), and when wax was activity being synthesized (days 30–61). Other species in the graphs are separated by color. For each species (except tomato) the average expression of the respective gene across a time-course was computed and the range of expression is represented by the vertical lines within each bar. (A) Ratio of transcripts for acyl-ACP thioesterases (FATB and FATA). (B) Ratio of transcripts for keto acyl-ACP synthase (KAS) I and KASII. (C, D) The expression of stearoyl-ACP desaturase (SAD) (C) and fatty acid desaturases (FAD) 2 and 3 (D), relative to the total number of transcripts associated with acyl-lipid metabolism (defined in reference 11).

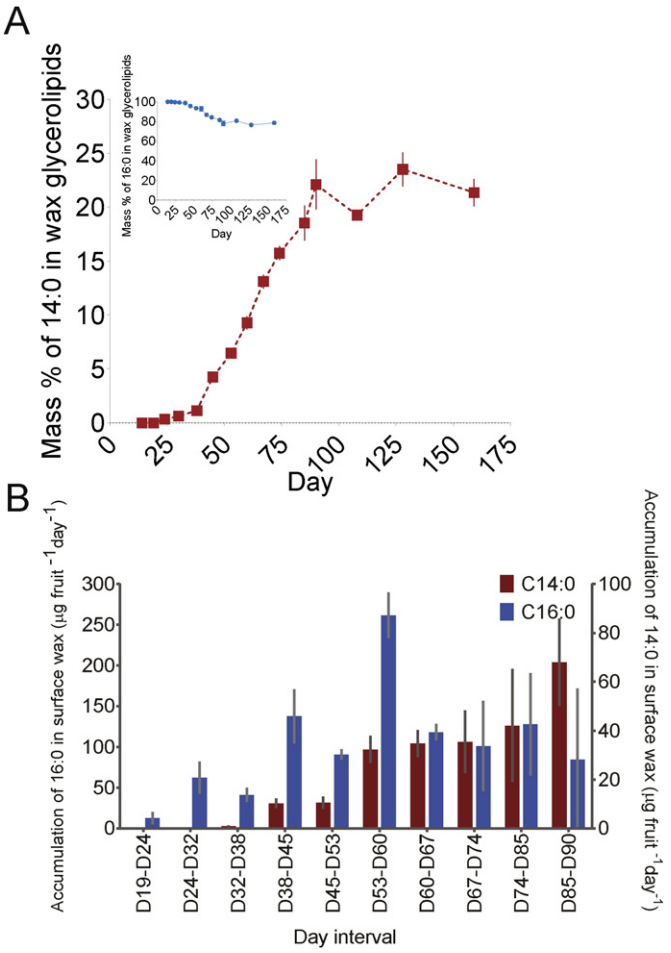

Fig. 4. The proportion of 14:0 fatty acids increases relative to 16:0 during Bayberry wax synthesis. (A) The mass percentage of 14:0 and 16:0 (insert) fatty acids in Bayberry wax glycerolipids through development. (B) Rate of synthesis, calculated from data in A, of 16:0 (left axis, blue bars) and 14:0 (right axis, red bars) in Bayberry wax glycerolipids through sampling intervals. Each point represents the average of 3–4 replicates ± SE.

the chain length distribution of the fatty acids in the glycerolipids changed during fruit development. At early stages, 16:0 represented greater than 98% of fatty acids incorporated into Bayberry wax. However, the relative abundance of the other major fatty acid in the wax, 14:0, increased through the season and reached 20–25% (with 75–80% 16:0) at maturity (Fig. 4A). Calculating the rates of accumulation of fatty acids in the surface wax throughout the season also illustrates an acceleration of 14:0 accumulation and the slowing of 16:0 accumulation, which was particularly evident after day 60 (Fig. 4B). In fact, during the final 5 days of wax synthesis, 14:0 accumulated in TAG and DAG at a similar rate to 16:0.

Because other plant species that accumulate large amounts of medium and short chain fatty acids express multiple isoforms of FATB, we asked whether the increase in 14:0 may be due to Bayberry knobs expressing multiple FATB transcripts with different specificities toward acyl-ACP structures [38,41]. However, in Bayberry knobs (and also leaves) only one transcript sequence was annotated as FATB. Instead, we considered that the increasing proportion of 14:0 fatty acids in the wax was related to flux through FAS because the highest rates of 14:0 accumulation coincided with an overall slowing of surface wax synthesis (evidenced by the decrease in accumulation of the dominant 16:0 fatty acid in the surface wax).

The proportions of 14:0 and 16:0 produced by FAS will be determined by the rate of 14:0-ACP hydrolysis relative to 14:0-ACP elongation by ketoacyl-ACP synthase (KAS) I. Increased production of C14 fatty acids, relative to C16, has been observed in plant extracts which are perturbed in different reactions of FAS [42]. For example, the proportion of 14:0 increases when FAS substrates malonyl–CoA and reductant (NADPH) are lowered, and also when ACP or acetyl–CoA is increased. The first two factors may directly affect the extension of 14:0 to 16:0, while the latter two examples may cause a saturation of FAS enzymes that favors premature termination of acyl-ACPs by thioesterases [45]. An additional factor influencing 14:0 production by Bayberry knobs may be that the ratio of FATB to KASI activity increases at later stages of development, which could increase the ability of FATB to "out-compete" KASI for the pool of 14:0-ACP substrate.

3.5. Reduced expression of transcripts for ketoacyl-ACP synthase II and fatty acid desaturases are associated with very high saturated fatty acid content in Bayberry wax

Consistent with the production of almost exclusively saturated fatty acids, Bayberry knobs expressed transcripts annotated as ketoacyl-ACP synthases (KAS) II and fatty acid desaturases at much lower relative levels compared to other plant species (Fig. 3B, C, D). KASII catalyzes the condensation of malonyl–ACP to acyl-ACP primarily for the elongation of 16:0 to 18:0 [11] while KAS I is the condensing enzyme for 4:0 to 16:0 elongation. In agreement with the low (1%) C18 and lack of unsaturated fatty acids in Bayberry wax, KASII transcripts were expressed at levels 10–20-fold lower than KAS I transcripts. In contrast, oil crops, (which produce more than 50% C18 fatty acids), *M. pensylvanica* leaves and *M. rubra* fruits express transcripts for KASII and KASI at approximately equal levels (Fig. 3B).

Transcripts for the plastid localized fatty acid desaturase (SAD), and the ER localized fatty acid desaturases (FAD2 and FAD3) were low in

Bayberry knobs, representing less than 2% of the sum of transcripts for all acyl-lipid genes. In contrast, other plants express much higher relative levels of transcripts encoding desaturase enzymes (Fig. 3C, D), in particular stearoyl–ACP desaturase. Thus, it is clear that in addition to high expression of FATB, which favors the production of 16:0 fatty acids, Bayberry knobs also have evolved mechanisms that down-regulate expression of transcripts for KASII and for the desaturases that produce and modify C18 fatty acids.

While Bayberry knobs appear highly specialized in their high expression of FATB and reduced expression of desaturases, the relative abundance of transcripts for pyruvate dehydrogenase, acetyl–CoA carboxylase and other genes associated with FAS was similar between Bayberry knobs and the other plant species analyzed here (Supplemental Figure 4). Similar consistent relative expression levels among FAS genes was previously noted among multiple different oil crops [13].

3.6. The initiation of Bayberry surface wax synthesis is associated with high expression of transcripts for specific cutin assembly genes

In contrast to the *sn*-1 acylation of glycerol-3-phosphate (G3P) for conventional intracellular glycerolipid synthesis, [^{14}C] labeling demonstrated that the first reaction for Bayberry glycerolipid assembly is the production of *sn*-2 MAG [3]. *Sn*-2 MAG is an early intermediate for cutin and its synthesis may be a branch point that separates fatty acyl chains for surface lipids from membrane lipids [7,43]. Furthermore, in vitro assays demonstrated that an extracellularly localized and cutin-associated GDSL-motif lipase/transacylase enzyme can catalyze the exchange of acyl-chains from *sn*-2 MAG to free ω-OH on another *sn*-2 MAG to synthesize a cutin-like polyester [8,44]. We proposed a model for Bayberry wax assembly that is related to current models for cutin assembly [7,45]. In particular, kinetic labeling of Bayberry knobs indicated that *sn*-2 may serve as the primary acyl-donor for DAG and TAG synthesis, and that TAG is assembled extracellularly [3].

The model for Bayberry surface wax assembly was proposed in part based on the very high expression of cutin-associated acyltransferase transcripts (*sn*-2 GPATs, DCR, and GDSL-motif enzymes) [3]. However, it was unclear whether Bayberry knobs are different in their high expression of transcripts for these acyltransferases compared to other plant tissues that actively accumulate abundant cutin. We therefore compared their expression in Bayberry knobs to homologous genes identified in the transcriptomes of tomato epidermis [15] and cherry fruits exocarp (i.e. epidermis of fruits) [16]. While the cutins of tomato and cherry are particularly abundant and are composed of 16:0-derived hydroxylated fatty acids, their rates of synthesis are over 10-fold less than the rate of wax deposition on Bayberry fruits [15,16]. Consistent with this large difference, the expression in Bayberry knobs of *sn*-2 GPATs and the homolog of Arabidopsis DCR were approximately 10-fold higher compared to tomato and cherry epidermal tissues (Fig. 5A, B). Of the transcripts encoding the highly expressed GDSL-motif enzymes in Bayberry knobs, only Mp-GDSL2, was expressed during the period of active wax deposition (days 30–51); however, neither tomato or cherry express genes closely related to Mp-GDSL2 at high levels (Fig. 5C, D). While the very high expression of Mp-GDSL2 in Bayberry knobs is suggestive of a specific role in its wax assembly, functional characterization of Mp-GDSL2 or its homologs (greater than 50% amino acid identity) from Arabidopsis or other species has not been reported.

These comparisons illustrate that transcript expression in Bayberry knobs is specialized in its very high expression of *sn*-2 GPATs, DCR, and GDSL-motif enzymes, even when compared to other fruit tissues that accumulate high cutin loads. This adds further support to the conclusion that these specific cutin-related acyltransferases are enzymes required for the synthesis of the abundant and unusual surface wax in Bayberry. We speculate that Bayberry knobs express those transcripts at a high level because the tissue requires very abundant enzymes to achieve high levels of production of surface lipids. However, we cannot rule out that high expression is also required because the enzyme catalytic sites may not have adapted to assemble glycerolipids with non-cutin (i.e. no hydroxy groups) fatty acyl groups.

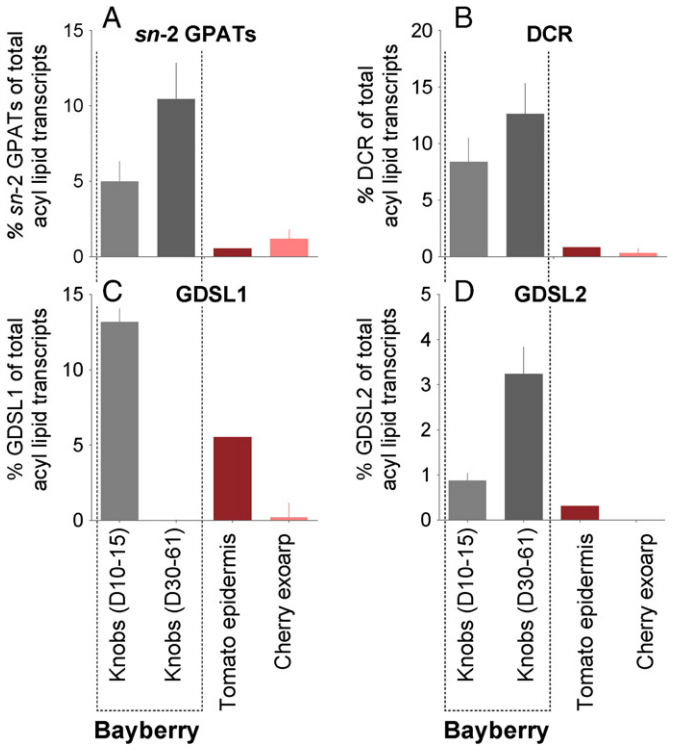

Fig. 5. Relative expression of cutin-associated acyltransferase/transacylase transcripts in Bayberry knobs is much higher than in tomato epidermis or cherry exocarp-enriched tissue. Bars indicate the expression of transcripts relative to total number of transcripts associated with acyl-lipid metabolism for (A) *sn*-2 GPATs, (B) DCR, (C, D) GDSL-motif enzymes. For Bayberry knobs, average expression (RPKM) was calculated for stages when wax was not visible (days 10–15) and when wax accumulation was active (days 30–61). For cherry exocarp, expression was averaged across a time-course. The error bars represent range.

3.7. Down-regulation of key cutin genes in Bayberry knobs may prevent cutin synthesis and favor the synthesis of the soluble glycerolipid wax

Bayberry knobs produce a cutin layer consisting primarily of dihydroxypalmitate (DHP) acyl chains (Supplemental Figure 5), which are derived from 16:0 fatty acids. Despite cutin and Bayberry surface wax sharing, a similar C16-based fatty composition, accumulating in the same location, and evidence that they share the same assembly enzymes, we could not detect any cutin-like hydroxy fatty acids or TAG estolides in Bayberry wax. This implied that Bayberry knobs have significantly down-regulated the flux of 16:0 fatty acids toward enzymes which contribute to the synthesis of cutin and instead diverted essentially all saturated fatty acids toward MAG, DAG, and TAG synthesis. While the very high expression of transcripts encoding cutin-associated genes *sn*-2 GPAT, DCR, and Mp-GDSL2 is associated with the synthesis of Bayberry wax, we also examined whether any lipid-related genes were specifically down-regulated, which may indicate a strategy to prevent the continued synthesis of the insoluble polyester cutin.

Cutin is deposited early in the development of a plant tissue [20]. The first two sampling stages for Bayberry knob RNA-seq were from very young fruits when surface wax was barely detectable (Supplemental Figure 2). At these stages, several transcripts related to cutin synthesis exhibited differential expression between the first two sampling stages

(i.e. no wax accumulation) and the final sampling stages (i.e. wax production) (Fig. 6, Supplemental Table 2). The expression of Mp-GDSL1 was the most striking case as it was one of the highest expressed transcripts during the first two sampling dates, but was one of the lowest expressed from days 30–51 when surface wax accumulation was greatest (Fig. 6B). Mp-GDSL1 is closely related to Cutin Deficient 1 (CD1) from tomato (76% amino acid identity) [3]. CD1 is one of the highest expressed enzymes in tomato fruit epidermis and mutant studies and in vitro assays indicate it catalyzes the extracellular assembly of cutin with *sn*-2 MAG as the acyl-donor [8,44]. In contrast to Mp-GDSL1, Mp-GDSL2 falls in a separate phylogenetic clade [3], and Mp-GDSL2 increased in expression (to within the top 20 most highly expressed genes) as wax synthesis was increasing. As discussed above, the disparate expression patterns of the two GDSL-motif enzymes suggest Mp-GDSL2 has a specific role for the assembly of DAG and TAG, while Mp-GDSL1 may contribute to cutin synthesis. However, because GDSL-motif enzymes are extracellular and are thermostable [44], it is possible that Mp-GDSL1 continues to be active in extracellular wax synthesis, despite the decrease in transcript levels.

Analogous to the expression of Mp-GDSL1, the expression of homologs to three cutin-associated acyl-chain-modifying enzymes were at their highest levels at the first two sampling times and then decreased in expression over 100-fold through the remaining sampling times (Fig. 6E). The Arabidopsis homologs for these Bayberry transcripts are the oxidoreductase HOTHEAD [46], and P450 hydroxylases CYP86A4 and CYP77A6/A4 [30]. In cutin synthesis, these enzymes modify newly synthesized fatty acids [7] to facilitate linkages (i.e. ester bonds) to other fatty acids creating the cutin polyester. It is unclear why a P450 hydroxylase annotated as CYP86A8/LACERATA(LCR) [47] increased in transcript abundance during the wax accumulation phase in Bayberry because no hydroxy fatty acids were detected in Bayberry MAG, DAG, or TAG.

In Bayberry knobs, the reduced expression of transcripts for homologs to cutin synthase/CD1 (i.e. Mp-GDSL1) and for acyl chain oxidizing enzymes contrasts sharply with elevated expression for Mp-GDSL2, *sn*-2 GPATs, and DCR (Fig. 6A, B). Transcripts for Bayberry *sn*-2 GPATs and DCR were also high at the first two sampling stages, when wax synthesis was barely detectable, suggesting that they may also be involved in the synthesis of knob cutin. Thus, it is possible that for Bayberry knobs to initiate glycerolipid wax synthesis, the cells uncouple the temporal regulation of cutin genes by down-regulating only a subset of genes involved in the synthesis of cutin (i.e. P450s, Mp-GDSL1), while greatly increasing expression of genes including *sn*-2 GPATs, DCR, and Mp-GDSL2. This coordination, compared to tissues actively synthesizing cutin, may allow Bayberry knobs to change its biochemistry to produce and secrete only soluble glycerolipids, containing no hydroxy fatty acids, after a cutin layer is deposited on the young fruits.

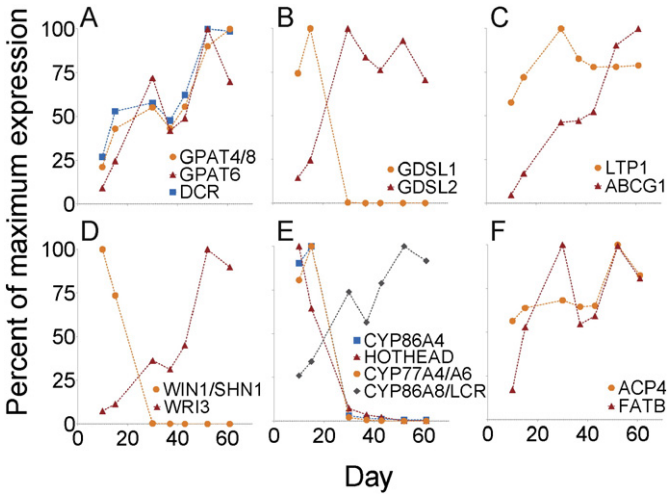

Fig. 6. Transcript expression profiles for selected acyl-lipid-associated genes in Bayberry knobs during development. Expression for each gene was calculated relative to their highest expression value during the RNA-seq time-course (set to 100). (A) Cutin assembly acyltransferases. (B) GDSL-motif enzymes highly expressed in Bayberry knobs. (C) Transport-associated proteins. (D) Transcription factors. (E) Cutin modification enzymes. (F) Abundant FAS genes.

3.8. ABCG transporter and lipid transfer proteins

ABCG transporters [7,48,49] and LTPs [50,51] contribute to the transport of cuticular waxes and cutin precursors through the cell membrane and cell wall, respectively, and were among the top 55 most highly expressed transcripts in Bayberry knobs. The most abundant ABCG transporter transcript in Bayberry knobs is closely related to Arabidopsis ABCG1 (Supplemental Figure 6). In contrast to Bayberry knobs, tomato and cherry ABCG transporters are not highly expressed (Supplemental Table 3). The expression of Bayberry ABCG1 increased 20-fold through development (Fig. 6C) and was expressed 3 to 5-fold higher than transcripts for the next highest expressed ABCG transporter.

The affinity of plant ABCG transporters for specific lipids has not been established and no evidence for a direct role in MAG export is available [52]. In other plants, ABCG1 proteins have been implicated in the production of suberin in potato tubers [48] and in Arabidopsis roots, seed coat, and pollen wall [49]. Assuming the highly expressed Bayberry ABCG1 participates in export of DAG and/or MAG from knob cells, this example implies this specific class of ABC transporter can transport a wide range of lipid structures.

The most highly expressed of any annotated transcript in Bayberry knobs was a lipid transfer protein homologous to Arabidopsis LTP1 (At3g28540) (Supplemental Figure 7). In addition to LTP1, two other classes of LTPs (annotated as type 2 with Arabidopsis homolog ID At3g18280 and At1g48750) were also within the top 55 most highly expressed genes, and the sum of LTPs represented over 2% of all annotated transcripts in Bayberry knob and up to 35% of total acyl-lipid-associated transcripts (Supplemental Table 3). LTPs are small (~9 kDa), soluble, extracellular proteins that are highly expressed by epidermal cells and are among the most abundant proteins on plant surfaces [53–55]. Specific functional roles have been difficult to assign to the different classes. However, LTP1 is believed to function in the secretion of hydrophobic substances from cells and mutant phenotypes indicate a role for the gene in stigmas, trichomes, and in roots that form symbiotic relationships with microorganisms [54,56,57]. Unlike ABCG transporters and most cutin assembly genes discussed above, LTP transcripts are also very abundant in tomato epidermis and cherry exocarp tissue. Thus, Bayberry knobs are not unusual in their very high expression of LTPs.

3.9. Have protein sequences of key enzymes evolved to produce Bayberry wax?

As described above, very high expression of a subgroup of cutin-related transcripts is associated with the synthesis of Bayberry wax. We asked what other mechanisms might have been required for Bayberry to produce wax chemical structures that are unique compared to conventional cuticular lipids. Commonly cited mechanisms for evolution of novel pathways in plants, particularly in specialized secondary metabolism, involve (1) gene duplication followed by neo-or subfunctionalization [58], or (2) broadening /altering enzyme specificity to allow for additional metabolic reactions [59]. For example, in Bayberry, a duplication of a *sn*-2 GPAT gene may have allowed for one protein to evolve the ability to prefer 16:0 fatty acids for wax synthesis while the other retained specificity for hydroxy fatty acids for cutin synthesis. We therefore examined the extent to which the protein sequences encoded by the very abundant transcripts may have evolved in Bayberry to achieve its unusual wax composition.

We compared the predicted consensus protein sequences encoded by acyl-lipid-related transcripts that were highly expressed in knobs to homologous sequences from leaf tissue collected from the same plants (Supplemental File 2). Bayberry leaf wax does not accumulate MAG, DAG, or TAG, and its cutin layer is predominantly composed of dihydroxy- and hydroxy-16:0 monomers (not shown). Surprisingly, the predicted protein sequences for 11 of the 13 highly expressed lipid genes in Bayberry knobs were 100% identical to sequences assembled from reads derived only from the leaves. All predicted biosynthetic enzymes were 100% identical between the tissues, and the two differences were in the putative transporters LTP2 (>95% identical between the tissues) and ABCG1 (which was partially truncated in the knobs). We also compared the same protein sequences in Bayberry knobs to Chinese Bayberry (*Myrica rubra*). *Myrica rubra* does contain knob-like structures on the surface of its fruits; however, they do not accumulate an abundant layer of wax similar to *M. pensylvanica* and the two species diverged from each other approximately 30 million years ago [60]. Despite that, the predicted protein sequences of *M. rubra* shared 90%–95% identity to *Myrica pensylvanica*. This finding strongly suggests that, at least for abundantly expressed genes, expression level, and not changes in protein sequence, was a primary mechanism for the evolution of surface wax on *M. pensylvanica* fruits.

3.10. Transcription factors

The major up-regulation and down-regulation of specific transcripts discussed above likely required changes in promoters and/or their transcription factors, or other non-coding DNA sequences that influence expression. We therefore also asked if altered expression of specific transcription factors (TF) may have contributed to the expression of the cutin-related gene transcripts in Bayberry knobs. Over 700 putative TFs (based on homology to Arabidopsis genes) were identified in Bayberry from RNA-seq (Supplemental File 3). Of particular interest were TFs that were abundantly and differentially expressed in the knobs relative to Bayberry leaves and *M. rubra* fruits. While it is possible that other highly expressed TFs may also have a role in wax synthesis, the two lipid-related transcripts that fit the criteria were homologs to Arabidopsis WRINKLED 3 (WRI3) (At1g16060) and MYB30 (At3g28910).

Arabidopsis WRI3 is closely related to WRINKLED 1 (WRI1), which regulates the expression of genes involved in glycolysis and fatty acid synthesis in seeds [61,62]. Indicative of a role in surface lipid synthesis, an Arabidopsis knockout in WRI3 caused a severe reduction in floral cutin and wax load but did not affect seed oil content. [63]. In Bayberry knobs, transcripts homologous to AtWRI3 increased 10-fold during wax accumulation (Fig. 6D) and was expressed 44-fold higher than leaves, and 9-fold higher than a WRI3 sequence identified in *M. rubra* fruits. Thus, WRI3's specific expression in Bayberry knobs suggests it contributes to the high fatty acid synthesis required to support the abundant wax production by Bayberry knobs. MYB30 was reported to be a positive regulator of hypersensitive cell death [64] but is a candidate for influencing Bayberry wax production because it was shown to up-regulate genes associated with cutin [65]. The putative Bayberry MYB30 was expressed 3-fold higher in knobs than Bayberry leaves and 9-fold higher than *M. rubra* fruits.

One transcription factor associated with surface lipids that was not highly expressed in the knobs and that did not show differential expression between the knobs and the leaves and *M. rubra* fruits were transcripts homologous to the WIN/SHINE. WIN/SHINE transcription factors in Arabidopsis and tomato bind directly to cutin-associated genes and overexpression and knockout of the transcription factor disrupted cutin synthesis [66,67]. Interestingly, much like Mp-GDSL1 and the cutin-associated hydroxylases that decrease in expression during wax accumulation, the expression of WIN/SHINE dropped substantially during fruit maturation (Fig. 6D, Supplemental Table 2). Thus, perhaps surprisingly considering its role in control of cutin-related gene expression, WIN/SHINE may not be directly involved in the synthesis of Bayberry surface wax.

4. Conclusions

Bayberry surface wax is an extraordinary example of specialized acyl-lipid metabolism in both quantity and quality of its products. The biosynthesis of DAG and TAG for Bayberry wax is clearly different from conventional intracellular glycerolipid synthesis in plants. Instead, multiple lines of evidence indicate that Bayberry knobs have evolved a novel pathway for TAG synthesis and that this was achieved in large part by "re-purposing" genes of cutin synthesis.

The goal of this study was to provide insights into the evolution of Bayberry's unusual surface lipid metabolism. Toward this, we examined the development, morphology, and subcellular anatomy of the lipid-secreting tissue (i.e. knobs), and also compared transcript profiles in knobs to plant tissues that share commonalities to the knobs (i.e. epidermal tissues of tomato and cherry fruit, oil palm mesocarp, *B. napus* embryos, Bayberry leaf, and fruits of the related species *Myrica rubra*). These comparisons revealed that Bayberry (1) secretes surface wax by mechanisms similar to conventional surface lipids, (2) modified the expression of FAS genes to favor the synthesis of saturated fatty acids, (3) expresses transcripts related to cutin assembly at very high levels, (4) strongly down-regulated transcripts for enzymes that modify cutin fatty acids (i.e. hydroxylases). Furthermore, (5) the predicted protein sequences for highly expressed enzyme-encoding transcripts exhibited no significant differences between knobs and leaves of the same plant and were very similar to *M. rubra* fruits. This leads to the perhaps surprising conclusion that the synthesis of surface DAG and TAG in Bayberry wax was achieved without changes in the protein sequences or specificity of key enzymes. Instead, the comparative analysis across species and tissues reveals that modified expression of a small subset of cutin and FAS genes may have been the major mechanism to establish the synthesis and extracellular accumulation of very large quantities of glycerolipids on Bayberry fruits.

Fig. 7 presents a model for Bayberry wax biosynthesis which extends the model proposed in Simpson and Ohlrogge (2016) [3] by proposing functions for the highly expressed genes: (1) the Bayberry FATB releases 16:0 and 14:0 fatty acids from FAS and contributes to the lack of 18:0 and desaturated fatty acids in the wax; (2) 16:0 (and 14:0) acyl-CoAs are transferred to G3P by the bi-functional *sn*-2 GPATs; (3) both the kinetics of radiolabeling and the positional distribution of radiolabeled acyl chains in DAG suggested that DAG is synthesized from *sn*-2 MAG by an intracellular MAG: MAG transacylase. (4) DAG and MAG are likely exported to the surface by the combination of ABCG1 transporters and LTPs; (5) the final step of TAG synthesis occurs outside of the cell catalyzed by extracellular GDSL-motif enzymes.

This model was developed based on predicted activities of the highly expressed transcripts and radiolabeling of Bayberry knobs [3]. Because many aspects of cutin synthesis remain unknown, and the localizations and activities of the highly expressed Bayberry enzymes could not be directly tested, the proposed model is hypothetical and there may be additional unknown reactions not shown in the figure. Of note, a role for DCR in cutin and Bayberry wax assembly has not been clearly established [68]. Since DCR is cytosolic and is the highest expressed transcript for any Bayberry enzyme, we propose that it is involved in the intracellular synthesis of DAG; however, we cannot yet rule out a scenario where DAG synthesis also, or perhaps exclusively, occurs outside of knob cells.

The function of Bayberry wax is most likely not as an energy/carbon store for the plant, but instead the wax may be an attractant to some species of birds for seed dispersal [6,69]. In that regard, Bayberry wax is analogous to the large accumulations of lipids seen in the fleshy mesocarps of oil palm, olive, and avocado. However, in contrast to Bayberry, those tissues up-regulated conventional fatty acid and TAG synthesis genes to produce large quantities of TAG and store it within, rather than

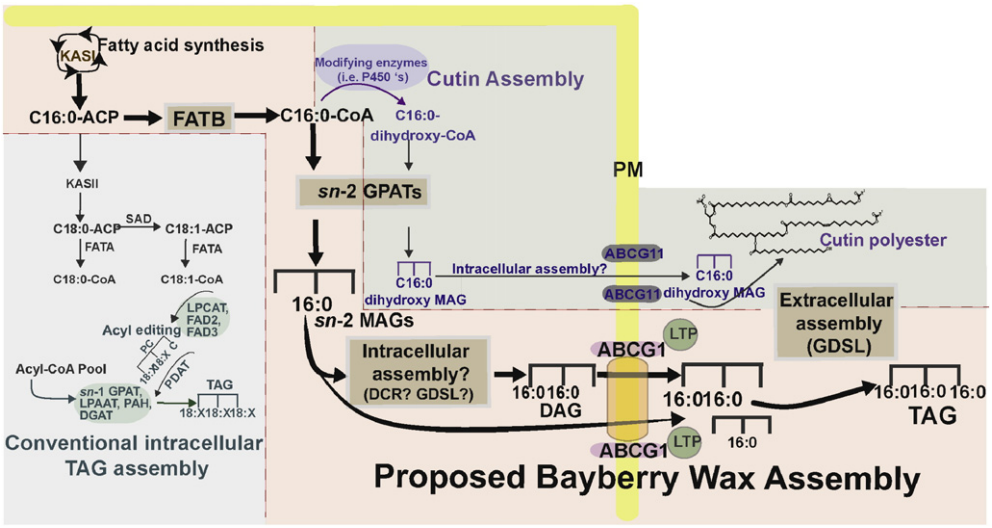

Fig. 7. Proposed Bayberry wax assembly pathway. Reactions in the model were predicted based on labeling and transcript expression data (see Simpson and Ohlrogge, 2016), and not all possible reactions are known or represented in the model. This schematic does not identify cellular structures, except for the plasma membrane (PM), denoted in yellow. Intracellular reactions are to the left of the PM, and extracellular reactions are to the right of the PM. Bayberry wax assembly is highlighted in pink and outlined by the dashed line. For comparison, abbreviated pathways for intracellular TAG synthesis (gray) and cutin assembly (purple/green) are shown. All three pathways begin with the synthesis of fatty acids (top left). For Bayberry wax, 16:0 and 14:0 fatty acids are released from FAS by FATB, activated, and then used for the synthesis of *sn*-2 MAG by *sn*-2 GPATs. Data strongly support DAG assembly by a MAG: MAG transacylation. The model proposes that DAG synthesis occurs inside knobs and possibly catalyzed by the enzyme defective in cuticular ridges (DCR); however, we cannot rule out an alternative scenario in which DAG synthesis occurs outside cells and the involvement of other enzymes. Next, DAG and a portion of the MAG are secreted from the cell through the highly expressed ABCG transporters. LTPs may also contribute to lipid secretion. Finally, extracellularly localized GDSL-motif enzymes participate in the assembly of TAG from MAG and DAG, which is analogous to cutin synthesis. In the cutin synthesis pathway shown (purple/green), 16:0 fatty acids are hydroxylated by P450 enzymes to produce hydroxy- and dihydroxypalmitate (DHP), which are utilized by *sn*-2 GPATs to produce *sn*-2 MAG, which is then secreted from cells and assembled into cutin by GDSL-motif enzymes (see eats et al., 2012 and Beisson et al., 2012). For the conventional intracellular TAG synthesis pathway (gray), only C18 fatty acid flux into TAG is shown.

outside the cells [14,70]. While Bayberry and oil-accumulating mesocarps may have experienced similar selection pressures for increased seed dispersal, Bayberry knobs have instead re-purposed enzymes associated with the synthesis of the glycerolipid cutin to accumulate extracellular TAG and DAG. This evolution apparently required mainly altered expression levels and not new enzyme specificity.

From a biotechnology perspective, could other plants be engineered to accumulate large amounts of DAG and TAG in extracellular waxes like Bayberry? Evidence here suggests that this might be achieved by altering the timing and expression levels of genes associated with the biosynthesis of cutin. It has already been demonstrated that ectopic overexpression of the suberin-associated GPAT5 results in *sn*-2 MAG surface accumulation [9], and overexpression of a suberin-associated transcription factor induces the synthesis and accumulation of suberin in leaf [71]. This indicates that epidermal lipid metabolism in plants can be remodeled to produce and secrete atypical lipids by simply overexpression of single enzymes or a transcription factor. Furthermore, results from Bayberry suggest plasticity in the ability of ABCG proteins to transport different types of lipids out of cells. This research on Bayberry surface DAG and TAG synthesis may thus prove useful in understanding alternative pathways to produce glycerolipids in plants and in particular for engineering plants to secrete high-value lipids that have toxic or negative consequences when accumulated in cells.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbalip.2016.01.022.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

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