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Leaf sheath cuticular waxes on bloomless and sparse-bloom mutants of *Sorghum bicolor*

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Abstract

Leaf sheath cuticular waxes on wild-type *Sorghum bicolor* were approximately 96% free fatty acids, with the C_{28} and C_{30} acids being 77 and 20% of these acids, respectively. Twelve mutants with markedly reduced wax load were characterized for chemical composition. In all of the 12 mutants, reduction in the amount of C_{28} and C_{30} acids accounted for essentially all of the reduction in total wax load relative to wildtype. The *bm*2 mutation caused a 99% reduction in total waxes. The *bm*4, *bm*5, *bm*6, *bm*7 and *h*10 mutations caused more than 91% reduction in total waxes, whereas the remaining six mutants, *bm*9, *bm*11, *h*7, *h*11, *h*12 and *h*13, caused between 35 and 78% reduction in total wax load. Relative to wild-type, *bm*4 caused a large increase in the absolute amount of C_{22}, C_{24} and C_{26} acids, and reduction in the C_{28} and longer acids, suggesting that *bm*4 may suppress elongation of C_{26} acyl-CoA primarily. The *h*10 mutation increased the absolute amounts of the longest chain length acids, but reduced shorter acids, suggesting that *h*10 may suppress termination of acyl-CoA elongation. The *bm*6, *bm*9, *bm*11, *h*7, *h*11, *h*12 and *h*13 mutations increased the relative amounts, but not absolute amounts, of longer chain acids. Based on chemical composition alone, it is still uncertain which genes and their products were altered by these mutations. Nevertheless, these *Sorghum* cuticular wax mutants should provide a valuable resource for future studies to elucidate gene involvement in the biosynthesis of cuticular waxes, in particular, the very-long-chain fatty acids. © 2000 Published by Elsevier Science Ltd.

Keywords: *Sorghum bicolor*; Cuticular wax; Fatty acid; Mutants; Acyl-CoA elongation

1. Introduction

Cuticular waxes form the outermost barrier over essentially all aerial plant surfaces and are among the most important traits for plant survival in stressful environments (Jenks and Ashworth, 1999). The most common plant waxes are very long chain aliphatic molecules, of mainly 16–34 carbons in length, that occur as either free fatty acids, aldehydes, primary alcohols, alkanes or esters (Bianchi and Bianchi, 1990). In the synthesis of fatty acids having 16 carbons or less, acyl chains are activated by a soluble plastidic acyl carrier protein (ACP) and elongated by a single fatty acid synthase (FAS) complex that condenses acetal groups from malonyl-ACP onto growing acyl-ACP chains (Ohlrogge and Jaworski, 1997). In contrast, the very long acyl chains that serve as direct wax precursors are activated by coenzyme-A (CoA) and synthesized by membrane-associated enzyme complexes called elongases (Agrawal et al., 1984). Elongases use malonyl-CoA as the two carbon donor instead of malonyl-ACP. Once synthesized, the very

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long acyl-CoA chains are converted to cuticular waxes after (1) deactivation by acyl-CoA thioesterases to release free acids (Liu and Post-Beittenmiller, 1995), (2) conversion to aliphatic esters by condensation of the acyl moiety with a primary alcohol by a putative acyl-CoA:fatty alcohol transacylase (Kolattukudy, 1967), or (3) entry into one of the two reductive pathways that either convert acyl-CoA to aldehydes and then primary alcohols, or convert acyl-CoA to aldehydes and then alkanes (Vioque and Kolattukudy, 1997). Thus, the activity and regulation of these acyl-CoA elongases define a central control point in overall plant cuticular wax biosynthesis since all cuticular waxes are derived from these reactions. Despite the importance of acyl-CoA elongation to wax synthesis, very little is currently known about how these very-long-chain fatty acyl-CoAs are elongated, or how their acyl moieties are then shunted to the various wax biosynthetic pathways.

Numerous explanations have been set forth to describe metabolic control over the elongation and chain-length-distribution of cuticular wax constituents. Chemical inhibitor studies were the first to suggest that numerous acyl-CoA elongase complexes condense acetal groups onto acyl-CoA chains in a chain-length-specific manner (Agrawal et al., 1984; Mikkelsen, 1978; Wettstein-Knowles, 1995). By comparison, genetic studies suggested that mutations in non-allelic genes coding for subunits of separate C_{24}, C_{26}, C_{28} and C_{30} elongase complexes explained reductions in chain-length-distributions of mutant wax constituents (Jenks and Ashworth, 1999). Other studies suggested that acyl chain lengths may be influenced more by chain-length-specific acyl-CoA thioesterases that terminate elongation by hydrolyzing acyl-CoA (Pollard et al., 1991; Voelker et al., 1997; Ohlrogge et al., 1978). Other factors that likely influence wax constituent chain length are the rate and chain-length-specificity for conversion of the acyl-CoA pool(s) by the two acyl-CoA reductases involved in primary alcohol and alkane synthesis, and the acyl-CoA transferase(s) involved in ester synthesis. Currently, the degree to which elongases, thioesterases, reductases, and transacylases influence or regulate acyl-CoA elongation and final wax constituent chain-length-distribution is unknown.

Based on preliminary studies, we hypothesized that mutants in *Sorghum* selected for visible differences in their waxy blooms should possess gene mutations that affect synthesis of the very-long-chain fatty acids primarily. Here, we report the composition of cuticular waxes on the wildtype and 12 cuticular wax mutants, and discuss the potential of *Sorghum* as a model system for elucidating gene involvement in reactions associated with acyl-CoA elongation and hydrolysis.

2. Results

2.1. Composition of filament and plate-like wax crystals on wild-type sheaths

Wild-type *Sorghum* sheaths had a glaucous coating and scanning electron microscopy (SEM) revealed that sheaths were covered by filamentous wax crystals of about 1 μm diameter (Fig. 1 (A) and (B)) that arise in groups from sites in the surface (Fig. 1(B)). Plate-like wax crystals coated the cuticle over all epidermal long cells (Fig. 1(B), see arrows). When wild-type sheath waxes were removed using chloroform (which removes both epi- and intra-cuticular waxes), fatty acids made 96% of the total waxes (Tables 1 and 2). The C_{28} and C_{30} acids comprised 77% and 20% of these acids, respectively (Table 3). The visible epi-cuticular waxes of wildtype were easily removed using gentle brushing with a toothpick. SEM revealed that this brushing removed a majority of the wax filaments, but caused

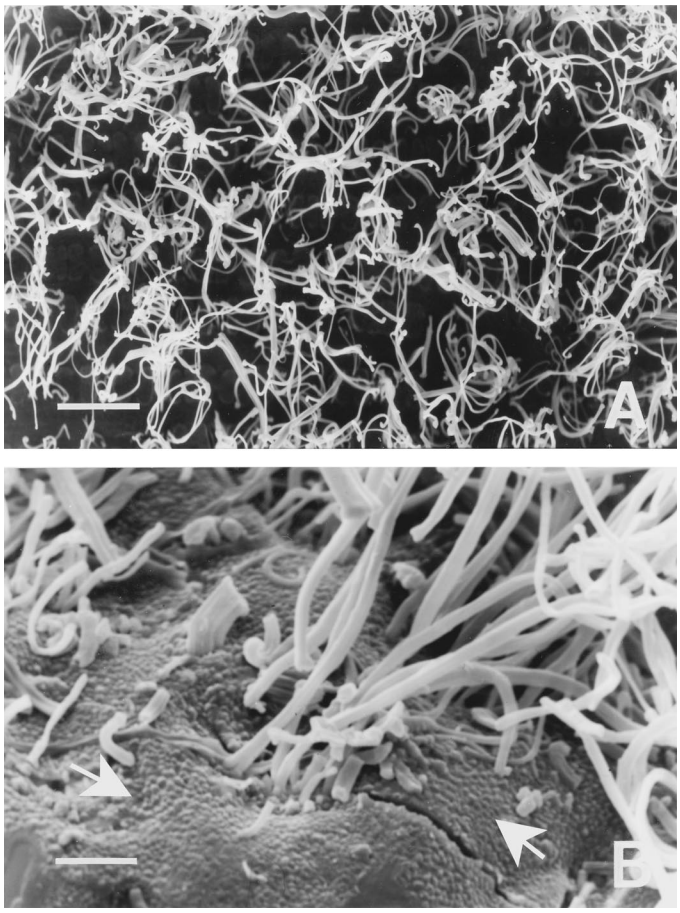

Fig. 1. Scanning electron micrographs showing microscopic surface morphology of air-dried abaxial leaf sheath surfaces of wild-type *Sorghum bicolor*. (A) View perpendicular to sheath surface showing emerging filamentous waxes. Bar = 20 μm. (B) View across the sheath surface showing filament wax attachment and plate-like waxes (arrows) over the entire cuticle surface. Bar = 5 μm.

Table 1
Composition of waxes from wild-type *Sorghum bicolor* and *bm*11 leaf sheaths isolated by chloroform extraction and/or brushing (wt% ± s.d.)[a]

	C_{28} acid	C_{30} acid	Other acids[b]	Non-acids
Wild-type	73.9 ± 5.1	19.2 ± 6.2	2.6 ± 0.6	4.3 ± 1.0
*bm*11	70.7 ± 6.9	20.2 ± 4.3	3.8 ± 1.3	5.3 ± 1.7
Filaments	77.9 ± 5.0	18.2 ± 6.5	0.3 ± 0.1	3.5 ± 1.6

[a] Wildtype and *bm*11 whole cuticular waxes were extracted using chloroform. Filaments were removed from wildtype using gentle brushing.
[b] Other acids indicates the total of C_{16}, C_{18}, C_{20}, C_{22}, C_{24}, C_{26}, C_{32} and C_{34} acids but does not include C_{28} and C_{30} acids.

little or no significant disturbance to plate-like waxes (not shown). Filaments removed using the brushing technique were not significantly different from wild-type chloroform extracts (*t*-test, $\alpha = 0.05$) (Table 1). The *bm*11 mutant is a bloomless type that produces no filament waxes from epidermal cork cells on its sheaths, however, it produces plate-like waxes over all long cells (Jenks et al., 1992). SEM micrographs suggested that *bm*11 does not secrete any form of wax from its cork cells. Although the total wax load was lower, the relative amounts of wax constituents on *bm*11 were not significantly different from wild-type chloroform wax extracts and isolated wild-type filaments (*t*-test, $\alpha = 0.05$; Table 1).

2.2. Amount and composition of cuticular waxes on sheaths of bm and h mutants

All mutants have less total sheath aliphatic cuticular waxes relative to the wild-type parent (Table 2). The unknown components (primarily triterpenoids) were all very minor components in wildtype and altogether accounted for less than 0.8% of the total waxes (not shown). No conspicuous change in these was evident on mutant wax chromatograms. The most altered mutant, *bm*2, caused a 99% reduction in total waxes. The *bm*4, *bm*5, *bm*6, *bm*7 and *h*10 mutations caused more than a 91% reduction in total waxes, whereas the remaining mutants, *bm*9, *bm*11, *h*7, *h*11, *h*12 and *h*13 caused between 35% and 78% reduction in total wax load (Table 2). Mutants were originally selected in the M_2 based on visible reductions in glaucousness of the abaxial sheath (Peters et al., 2000). While reduction in visible waxes can be generally related to reduction in total wax amount, this was not true for all mutants. For example, the bloomless mutants, *bm*9 and *bm*11, had much more wax than the sparse-bloom *h*10 mutant (Table 2), even though they had sheaths that were glossy green.

The reduction in the amount of acids accounted for essentially all the reduction in total wax load on all mutants (Tables 2 and 3). Proportionately, acid profiles produced using BSFTA derivatization of waxes extracted from greenhouse plants (Table 3) were nearly the same as those found with field-grown plant acids derivatized to FAMEs (not shown). One notable difference was that the field grown plants had lower total wax loads, likely due to degradation by wind and rain. In *bm*4, the C_{22} acid was the major acid constituent, followed by the C_{24} and C_{26} acids (Table 3). All three of these shorter chain acids occurred in higher absolute amounts on *bm*4 than in wild-type. By comparison, the C_{28}, C_{30}, C_{32} and C_{34} acids were much lower than in wildtype (Table 3). The *bm*2, *bm*5, *bm*6 and *bm*7 mutants had absolute reductions in essentially

Table 2
Amounts of fatty acids vs. other wax components isolated from the abaxial leaf sheaths of wild-type *Sorghum bicolor* and 12 isogenic cuticular wax mutants (μg/cm^2 ± s.d.)[a]

Genotype	Total load	Fatty acids	Non-acids
Wild-type	113.4 ± 15.4	108.5 ± 14.5	4.9 ± 1.4
*h*13	74.1 ± n.d.	68.7 ± n.d.	5.4 ± n.d.
*h*7	65.7 ± n.d.	62.5 ± n.d.	3.2 ± n.d.
*h*11	63.4 ± n.d.	58.5 ± n.d.	4.9 ± n.d.
*h*12	35.4 ± n.d.	33.4 ± n.d.	2.0 ± n.d.
*bm*9	25.4 ± 4.8	23.2 ± 4.7	2.2 ± 0.2
*bm*11	25.3 ± 4.3	23.9 ± 4.3	1.4 ± 0.3
*h*10	10.1 ± 0.8	8.9 ± 1.1	1.2 ± 0.2
*bm*4	9.1 ± 0.3	8.4 ± 0.9	0.7 ± 0.04
*bm*6	5.1 ± 0.5	4.2 ± 0.6	0.9 ± 0.2
*bm*5	3.5 ± 1.4	1.8 ± 0.9	1.7 ± 0.04
*bm*7	1.7 ± 0.7	1.1 ± 0.4	0.6 ± 0.3
*bm*2	0.6 ± 0.1	0.4 ± 0.6	0.2 ± 0.06

[a] n.d. is not determined. Only two replicates were analyzed for *h*7, *h*11, *h*12 and *h*13. Their range for total loads deviated 7%, 3%, 9%, and 12%, respectively, from the mean.

Table 3
Fatty acid profiles of wax from leaf sheaths of wild-type *Sorghum bicolor* and 12 isogenic cuticular wax mutants (μg/dm^2)[a]

Genotype	Chain length									
	16	18	20	22	24	26	28	30	32	34
Wild-type	14.2	6.8	6.1	43.1	63.2	141.9	8342	2218	12.1	3.1
*h*13	6.6	4.8	3.8	29.3	42.7	73.3	4964	1717	27.4	2.8
*h*7	11.7	7.9	6.0	30.5	43.5	42.0	4734	1351	11.2	1.5
*h*11	13.9	4.8	5.1	34.9	47.1	58.2	4835	1271	10.1	1.9
*h*12	6.0	3.8	2.2	13.7	22.9	34.3	2500	728.4	25.7	2.0
*bm*9	5.1	2.8	2.1	14.5	20.9	16.4	1577	660.1	10.5	1.5
*bm*11	5.9	3.2	2.9	14.7	21.0	33.3	1805	497.6	8.1	1.6
*h*10	7.0	2.7	0.8	5.2	10.4	5.4	194.8	604.4	59.0	9.1
*bm*4	4.4	1.7	2.4	325.0	261.6	207.0	30.7	1.3	0.8	0.5
*bm*6	10.6	5.9	3.4	8.9	19.5	5.5	228.7	129.1	5.5	2.8
*bm*5	33.8	15.3	5.2	18.9	40.3	8.9	51.1	10.1	tr	tr
*bm*7	12.6	8.6	5.1	7.7	14.0	4.9	31.2	19.2	3.4	0.1
*bm*2	3.3	2.0	1.3	3.5	7.1	2.0	12.5	3.7	tr	tr

[a] tr is trace amount.

all acid constituents, except for *bm*5 that had elevated C_{16} and C_{18} acids (Table 3). In relative amounts, *bm*2, *bm*5, *bm*6 and *bm*7 also had slightly greater reductions in C_{28} and C_{30} acids than in C_{16} through C_{26} acids (derived from Table 3). The *h*10 mutant had reductions in absolute amounts of all acids except for the C_{32} and C_{34} homologues (Table 3). Relative to wildtype, the *h*10 C_{20} through C_{28} acids were reduced, whereas the C_{30}, C_{32} and C_{34} acids were elevated (Table 3). The *h*12 and *h*13 acid profiles had reductions in the absolute amount of all acid constituents except the C_{32} acid (Table 3). The *bm*9, *bm*11, *h*7 and *h*11 mutants were similar except that their absolute amounts of C_{32} acids were equal or just slightly less than wild-type levels (Table 3).

The aldehyde, primary alcohol, alkane and ester constituents were also analyzed (Tables 4–7). Aldehydes were not detected on *bm*4 using both GC-MS (Table 4) and thin-layer chromatography (not shown). On all other mutants, aldehydes were lower than in wildtype except for *h*11 and *h*13 that had slightly higher C_{28} aldehydes than wildtype, and *bm*5 and *h*13 whose C_{26} aldehydes changed little, and *bm*9 whose C_{28} aldehydes likewise changed little (Table 4). The absolute amounts of primary alcohol constituents were markedly reduced below those of wildtype in all mutants, except *h*7 and *h*13 (Table 5). Like its acids, the *bm*4 primary alcohols were enriched in shorter length constituents with an increase in absolute amounts of the C_{22}, C_{24} and C_{26} homologues, and a decrease in the C_{28}, C_{30}, and C_{32} homologues (Table 5). In *h*13 mutant, amount of C_{24} through C_{30} alcohols changed little from wildtype, whereas in *h*7, the C_{28} alcohol was elevated slightly above wildtype (Table 5). The *bm*6 and *h*11 mutants showed little change in the C_{24} and C_{26} alcohols, respectively, whereas in *bm*5, C_{24} alcohols were slightly elevated (Table 5). The absolute amounts of all alkane constituents were reduced below wildtype in all mutants except *bm*5, *h*11 and *h*13 (Table 6). In these mutants, the C_{27} and C_{29} homologues occurred at slightly greater amounts (Table 6). Like its acid constituents, the *h*10 alkanes had proportionately more of the longer chain length homologues than wildtype, but reduced amounts of shorter homologues (Table 6). By comparison, an increase in the relative chain-length-distribution of *h*10 alcohols was not detected (Table 5). Esters were 0.1% of the total leaf sheath wax load (not shown). Ester loads on wildtype were 8.6 μg/dm^2 whereas mutants ranged between 3.3 μg/dm^2 for *bm*2 and 16.2 μg/dm^2 for *bm*5.

Table 5
Primary alcohol profiles of wax from leaf sheaths of wild-type *Sorghum bicolor* and 12 isogenic cuticular wax mutants (μg/dm^2)[a]

Genotype	Chain length					
	22	24	26	28	30	32
Wildtype	2.2	3.3	9.4	248.8	80.8	6.8
*h*13	0.6	4.5	11.1	276.5	95.4	3.1
*h*7	0.8	1.5	9.4	331.7	80.1	1.4
*h*11	0.9	2.5	10.3	217.3	55.3	3.6
*h*12	0.4	3.2	7.4	82.1	51.1	2.3
*bm*9	1.0	2.6	4.6	90.8	24.7	1.5
*bm*11	0.9	1.6	4.3	60.2	18.5	1.4
*h*10	0.9	1.3	3.6	33.6	19.2	1.2
*bm*4	10.0	14.9	16.7	6.1	1.9	0.2
*bm*6	1.6	3.5	5.3	23.0	10.1	0.5
*bm*5	0.6	5.8	25.5	33.8	3.4	tr
*bm*7	1.4	2.6	5.9	14.6	6.0	0.4
*bm*2	0.7	1.1	3.5	6.8	1.6	tr

[a] tr is trace amount.

Table 4
Aldehyde profiles of wax from leaf sheaths of wild-type *Sorghum bicolor* and 12 isogenic cuticular wax mutants (μg/dm^2)[a]

Genotype	Chain length			
	24	26	28	30
Wild-type	1.8	2.2	47.2	22.6
*h*13	1.7	2.6	73.4	13.7
*h*7	0.1	1.0	4.9	1.6
*h*11	1.6	2.2	86.1	25.0
*h*12	0.2	0.8	14.5	5.8
*bm*9	0.5	1.6	47.1	6.2
*bm*11	0.4	1.4	17.6	2.7
*h*10	0.2	1.2	2.0	4.8
*bm*4	0.0	0.0	0.0	0.0
*bm*6	0.1	0.5	5.2	2.5
*bm*5	tr	2.8	6.1	tr
*bm*7	tr	0.3	1.1	0.3
*bm*2	0.3	0.5	0.6	0.1

[a] tr is the trace amount.

Table 6
Alkane profiles of wax from leaf sheaths of wild-type *Sorghum bicolor* and 12 isogenic cuticular wax mutants (μg/dm^2)[a]

Genotype	Chain length					
	23	25	27	29	31	33
Wild-type	1.8	3.5	22.7	21.6	5.5	1.8
*h*13	0.1	4.0	30.9	39.8	5.5	1.6
*h*7	0.1	1.7	36.6	35.7	7.3	1.2
*h*11	0.1	3.5	26.8	27.9	9.4	1.4
*h*12	0.1	0.2	8.0	9.8	6.3	1.3
*bm*9	0.1	1.2	15.6	14.5	3.3	1.0
*bm*11	0.3	1.0	6.6	6.2	2.4	0.3
*h*10	0.2	1.0	5.8	19.5	5.9	2.2
*bm*4	1.0	1.6	4.3	3.6	1.3	0.5
*bm*6	0.3	1.4	11.4	14.4	3.2	1.3
*bm*5	0.9	4.1	36.7	29.1	2.4	tr
*bm*7	0.1	1.2	8.0	9.9	2.9	0.5
*bm*2	0.4	0.7	3.5	3.6	1.0	0.4

[a] tr is the trace amount.

There was relatively high standard deviation in ester load values and statistically significant differences between wildtype and mutants were not detected by our analyses (not shown).

3. Discussion

We previously reported that two separate epidermal cell types produced different types of epi-cuticular wax crystals on *Sorghum* leaf sheaths (Jenks et al., 1994b). Epidermal cork cells produced tubular filament wax crystals whereas epidermal long cells produced plate-like waxes. Wax filaments secreted by cork cells were visibly reflective, whereas long cell wax crystals were not. Since filaments and plates were distinct morphologically, we hypothesized that they may also have unique chemical composition. However, results here show that filaments are likely very similar in composition to plates. Only small differences existed between the relative amounts of chloroform extracted wild-type sheath waxes representing both plates and filaments, *bm*11 sheath waxes representing plates but no filaments (or any other cork cell secretions), and the isolated filament waxes from wildtype removed by gentle brushing. Previously, Jenks et al. (1994b) showed that wild-type *Sorghum* wax filaments were secreted from the apical wall of epidermal cork cells in association with specialized cell wall modifications called papillae, but no cell wall modifications were associated with secretion of plate waxes. The lack of any cork cell wax secretions in *bm*11 mutants was due to a mutation inhibiting cork cell specific wax biosynthesis (Jenks et al., 1994b). Although *bm*11 appeared to have normal cork cell papillae in these studies, further electron microscopy using chemically-fixed tissues would be needed to verify this. Regardless, differences in filament and plate wax morphology are likely due to differences in secretion site morphology of different epidermal cells (i.e. cork or long cells), and not due to large differences in chemical composition per se. Nevertheless, since proportionately small amounts of wax constituents can significantly influence wax crystal shape (Jetter and Riederer, 1995), large effects by minor constituents cannot be ruled out. Moreover, since cork cell filaments and long cell plates have similar composition, cork and long cells likely possess similar basic enzymatic pathways for wax synthesis. Whether cork cells produce more total waxes than long cells has yet to be determined.

Table 7
Nomenclature of bloomless (*bm*) and sparse-bloom (*h*) wax mutants derived from wild-type line P954035 identified by test of allelism in Peters et al. (2000). It should be noted that these designations are different than those assigned after initial screening and published characterizations (Jenks et al., 1992, 1994a, 1994b)

Locus and allele number	Original designation
*bm*2-3	*bm*22
*bm*4-2	*bm*15
*bm*5-1	*bm*16
*bm*6-1	*bm*21
*bm*7-1	*bm*26
*bm*9-1	*bm*31
*bm*11-1	*bm*38
*h*7-1	*bm*11
*h*10-1	*bm*28
*h*11-1	*bm*17
*h*12-1	*bm*24
*h*13-1	*bm*34

The wild-type sheath wax composition reported here resembled the values previously reported by Bianchi et al. (1978), except that in our studies, wild-type aldehydes and esters were proportionately lower. Further studies are needed to determine whether the differences in environment, genotype or extraction method between these studies explain these differences.

All 12 mutants reported here had much lower total wax load than wildtype leading us to believe that the genes altered by these mutations have important functions in wax biosynthesis. In all of 12 mutants, reduction in the amount of C_{28} and C_{30} acids accounted for nearly all of the reduction in total wax load relative to wildtype. Thus, these mutations occur in genes whose products play an important role in synthesis of the free C_{28} and C_{30} acids.

The *bm*4 mutation appeared to suppress elongation of C_{26} acyl-CoA primarily since the C_{22}, C_{24} and C_{26} acids were elevated above wild-type levels, the C_{28}, C_{30}, C_{32} and C_{34} acids were reduced below wildtype, and the C_{26} primary alcohol accumulated the most within the alcohol fraction (indicating that C_{26} alcohol precursors were most abundant). Similarly, the *cer*2 and *cer*6 mutations in *Arabidopsis* (Jenks et al., 1995), *gl*3 in *Brassica* (Macey and Barber, 1970a), and *wb* in pea (Macey and Barber, 1970b) all appeared to have their primary blockage at C_{26} acyl-CoA elongation since chain lengths of primary alcohols and alkanes on these mutants were dominated by constituents arising from 26 carbon-length precursors. In *bm*4, it is still unclear why the C_{26} acids did not accumulate more than the C_{22} and C_{24} acids, as might be predicted by a simple block in C_{26} acyl-CoA elongation.

Of the mutants analyzed, *bm*2, *bm*4, *bm*5, *bm*6 and *bm*7 had the largest reduction in amounts of all waxes at more than 92% below wild-type levels. Possibly, the *bm*4 mutant had reduced total wax load due to some form of feedback inhibition, perhaps by the increased shorter-chain acids (C_{22}, C_{24}, C_{26}). However, further studies are needed. Relative to wildtype, the shorter-chain acids on *bm*2, *bm*5 and *bm*7 were increased proportionately, but not in absolute amounts, relative to

other acids. Thus, it is still unclear why the total flux into the wax pathway was so greatly reduced in these mutants. Interestingly, pleiotropy by the *bm*2 mutation leads to nearly five-fold reduction in the weight of the cuticle proper, a membrane thought to be composed mostly of cutin biopolyester (Jenks et al., 1994a). Cutin synthetic enzymes use C_{16} and C_{18} acyl-CoA pools as precursors, potentially the same precursors used in wax synthesis. Thus, a reduction in common C_{16} and C_{18} precursor pools might explain how *bm*2 reduced both waxes and cuticle proper. Future studies using isotope labeling are needed to provide a more accurate prediction of precursor pool sizes in these mutants.

In absolute amounts, *h*10 produced 5.9-fold more C_{32} acid and 2.8-fold more C_{34} acid than wildtype, and had much higher relative amounts of the C_{30}, C_{32} and C_{34} acids than any other line. One possible explanation for the *h*10 acid profile was that its mutation suppressed C_{28} acyl-CoA hydrolysis by an acyl-CoA thioesterase, thereby preventing the deactivation of acyl-CoA and termination of acyl-CoA elongation. Suppression of acyl-CoA hydrolysis was likewise implicated in the longer chain-length-distribution of wax constituents on *cer*3 and *cer*7 in *Arabidopsis* (Jenks et al., 1995; Jenks, unpublished). Further studies are needed to determine whether *h*10 affects acyl-CoA thioesterase activity.

Relative to wildtype, the *bm*6, *bm*9, *bm*11, *h*7, *h*11, *h*12 and *h*13 mutations increased the relative amounts, but not absolute amounts, of the C_{30}, C_{32} and C_{34} acids. These mutations produced otherwise quite different phenotypes, with three of these mutants being bloomless and four sparse-bloom. In addition, the range for wax load reduction varied in these lines between 35 and 96%. Based on the chemical composition alone, it is still uncertain which gene products were altered by these mutations.

Interestingly, *bm*5 and *h*12 were slightly dwarfed, and had leaves that were more upright and brittle (i.e. they snapped easier when bent). It has been proposed that mutations affecting cell wall composition or architecture could influence the wax profile via effects on secretion of wax precursors from the epidermal cytoplasm to the cuticle surface (Jenks et al., 1996). Thus, *bm*5 and *h*12 might suppress acid synthesis by inhibiting the flow of acid precursors via their suppression of wax secretory pathways. Further studies are needed.

Based on wax compositional analysis in these 12 wax-deficient *Sorghum* mutants, all appeared to have lesions that altered either acyl-CoA elongation reactions, acyl-CoA hydrolysis, and/or precursor flow into or through the elongation pathway. Currently, only one or a few mutant alleles exist at each of the 24 known blomless (*bm*) and sparse-bloom (*h*) loci in *Sorghum* (Peters et al., 2000), suggesting that the genome of *Sorghum* has not yet been saturated for mutant loci affecting *Sorghum* sheath waxes. We believe that the *Sorghum* cuticular wax mutants reported here will provide a valuable resource for future studies to elucidate gene involvement in the biosynthesis of cuticular waxes, in particular, the very-long-chain fatty acids.

4. Experimental

4.1. Plant material

Based on the designations and tests for allelism defined by Peterson et al. (1982), the cuticular wax mutants analyzed in this study were designated by Peters et al. (2000) as either *bm* if all visible waxes were missing from the abaxial sheath surface, and *h* if visible waxes were reduced relative to wildtype but not completely missing. These *bm* and *h* mutants were derived by chemical mutagenesis, employing either diethyl sulfate or ethyl methanesulfate, on an elite pure line from the Purdue *Sorghum* Improvement Program. Bloomless and sparse-bloom mutants *bm*2-3, *bm*4-2, *bm*5-1, *bm*6-1, *bm*7-1, *bm*9-1, *bm*10-1, *bm*11-1, *h*7-1, *h*10-1, *h*11-1, *h*12-1 and *h*13-1 examined here are isogenic derivatives of the wild-type pure line P954035. In this paper, the allele number has been omitted from the mutant designation. All epi-cuticular wax mutants were selected using a visual screen for reduced leaf sheath surface glaucousness in the M_2 generation. Test of allelism and verification of single, nuclear gene inheritance were performed primarily in the M_4 generation (Peters et al., 2000). Based on these allelism tests, the new loci designations for the mutants analyzed here are presented (Table 7). These new loci designations should be applied to previous studies which employed the original designations (Jenks et al., 1992, 1994a, 1994b).

4.2. Scanning electron microscopy

Replicated, air-dried abaxial leaf sheath samples from greenhouse-grown wild-type *Sorghum* were mounted on aluminum stubs and sputter coated with gold palladium using short, 30 s bursts from the sputter coater. Previous research showed that air-dried samples coated in this way were similar to specimens prepared using low temperature scanning electron microscopy with little evidence of artifacts observed (Jenks et al., 1992). Coated surfaces were viewed using a JEOL JSM-840 scanning electron microscope (JEOL, Tokyo) at 10 kV.

4.3. Wax extraction

Epi-cuticular waxes from leaf sheaths (the vertical part of the leaf covering the culm) were extracted from the seventh through tenth leaf of wild-type and mutant lines grown in the greenhouse. Fully exposed sheath samples were collected using a #7 cork borer with circular area calculated as 1.389 cm^2. Sheath samples (5–20 disks depending on estimated wax load for each isoline) were inserted into a 20-ml standard glass scintillation vial and approximately 10 ml of GC grade chloroform added. The sheath tissues were agitated for 10 s and the solvent decanted off into new scintillation vials. Tissues and vials were given a 1 s rinse with approximately 2 ml of chloroform and then the solution decanted into the sample vial. The extracts thus contain waxes from both the abaxial and adaxial sheath surface, and also showed little or no coloration due to chlorophylls or other internal lipids. The adaxial surface was adpressed to the subtending sheath and not yet exposed to light. Since only very small amounts of wax are produced by sheaths before exposure to light (Jenks et al., 1994b, not shown), we assumed that our extracts were essentially pure abaxial waxes with only very small amounts of wax from the adaxial surface. Scanning electron microscopy revealed that no visible wax crystals were present on adaxial surfaces (data not shown).

4.4. Chemical analysis of cuticular waxes

Based on Jenks et al. (1995) study, the chloroform-soluble cuticular wax extracts were evaporated to dryness under a nitrogen stream and the dried residue prepared for gas chromatography by derivatization using BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide). Derivatization was for 15 min at 100°C. After surplus BSTFA was evaporated under nitrogen, the sample was redissolved in chloroform for analysis with a Hewlett-Packard 5890 series II gas chromatograph (GC) equipped with a flame ionization detector and automatic injector. The GC was equipped with a 12 m, 0.2 mm HP-1 capillary column with helium as the carrier gas. The GC was programmed with an initial temperature of 80°C and increased to 260°C at 15°C/min, where the temperature remained unchanged for 10 min. The temperature was then increased to 320°C at 5°C/min, where the temperature was held for 15 min. Quantification was based on flame ionization detector peak areas and the internal standard hexadecane that was added to the original extract. Conversion factors for detector units to amount values were developed from external standards of the free fatty acids, primary alcohol, and alkanes representing short to long chain homologues from all classes. These were analyzed at four concentration levels (multi-level) representing the concentration range of sample peaks. For aldehydes, a factor of 1.03 was assigned (the average conversion factor for all external standards). The total amount of cuticular wax was expressed per total abaxial plus adaxial leaf sheath area. All the values represent the average of two or four replicate samples. Wax esters were identified using TLC and cleaved and derivatized with methanol:acetyl chloride (10:1, w/v) into fatty acid methyl esters and primary-alcohol products. Selected sub-samples were used for injection in a gas chromatograph-mass spectrometer to produce electron ionization mass spectra for positive identification of all GC peaks.

4.5. Verifying fatty acid profiles

To verify fatty acid profiles, we performed a second acid analysis based on the creation of methyl-esters from fatty acids recovered from TLC and column chromatography. In this protocol, fatty acids were isolated from total surface waxes by passing 20–30 mg of the sheath wax sample over 5 cm of silicic acid treated with NaOH supported by a glass wool plug in a 0.3 cm ID champaign column (Supelco, Bellefonte, PA). Acids bind to the alkaline silica while neutral lipids wash through with the 50 ml of hexanes:diethyl ether (70:30 v/v) applied to the column. The column wash was filtered through a defatted Whatman 50 filter paper supported in a glass funnel, and then concentrated to dryness under nitrogen. Fatty acids were recovered from the silica gel by acidifying the column contents in a 10 N solution of sulfuric acid then extracting from the aqueous phase with diethyl ether (Wettstein-Knowles, 1974). Non-acid fractions were also partially examined as a means to verify results from mixed sample GC analysis. Separation efficiency was determined by monitoring recovered acid and neutral fractions by TLC on silica gel G using benzene as the developing solvent and concentrated sulfuric acid with charring for detection of lipids on developed plates. This TLC method was found previously to resolve the major lipid components of *Sorghum* surface waxes (Wettstein-Knowles et al., 1984).

The relative amount of free acids in the sheath cuticular wax was determined by weighing the neutral fraction which was recovered from the column wash. To correct for losses which occurred during the column chromatography procedure, a series of synthetic mixtures of known amounts of acid and neutral fractions derived from *Sorghum* wax were put through the procedure and a correction factor was thus obtained and included in all calculations. Acids were methyl esterified by reacting 0.1 mg of the acid fraction with 0.1 ml methanol:acetyl chloride (10:1 v/v) at 100°C for 10 min. The resulting mixtures of fatty acid methyl esters (FAME) were concentrated under nitrogen and

placed overnight in a desiccator. The dried products were redissolved in 50 μl chloroform and 1 μl of the mixture was then injected onto the column using the temperature program, 100–300°C at 10°C/min.

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