Composition of secondary alcohols, ketones, alkanediols, and ketols in *Arabidopsis thaliana* cuticular waxes

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Abstract

*Arabidopsis* wax components containing secondary functional groups were examined (i) to test the biosynthetic relationship between secondary alcohols and ketols and (ii) to determine the regiospecificity and substrate preference of the enzyme involved in ketol biosynthesis. The stem wax of *Arabidopsis* wild type contained homologous series of C27 to C31 secondary alcohols (2.4 µg cm⁻²) and C28 to C30 ketones (6.0 µg cm⁻²) dominated by C29 homologues. In addition, compound classes containing two secondary functional groups were identified as C29 diols (0.05 µg cm⁻²) and ketols (0.16 µg cm⁻²). All four compound classes showed characteristic isomer distributions, with functional groups located between C-14 and C-16. In the *mah1* mutant stem wax, diols and ketols could not be detected, while the amounts of secondary alcohols and ketones were drastically reduced. In two *MAH1*-overexpressing lines, equal amounts of C29 and C31 secondary alcohols were detected. Based on the comparison of homologue and isomer compositions between the different genotypes, it can be concluded that biosynthetic pathways lead from alkanes to secondary alcohols, and via ketones or diols to ketols. It seems plausible that MAH1 is the hydroxylase enzyme involved in all these conversions in *Arabidopsis thaliana*.

Key words: Chain lengths, cytochrome P450, fatty acid derivatives, gas chromatography, hydroxylation, MAH1, mass spectrometry, neutral lipids, plant surface composition, wax.

Introduction

The surfaces of aboveground, non-woody plant organs are covered with a cuticle, i.e. a thin hydrophobic membrane consisting of cutin and waxes. Cutin is thought to reinforce the cuticle structure mechanically (Stark and Tian, 2006), while waxes exert the primary physiological function of the cuticle to limit non-stomatal water loss (Schönherr, 1976). The cuticle is also of ecological importance as it forms the interface between plant organs and their environment. The hydrophobicity of cuticular waxes renders plant surfaces water repellent, thereby keeping them dry, guarding them from accumulation of particles, and preventing germination of pathogen spores (Holloway, 1970; Deising *et al.*, 1992; Barthlott and Neinhuis, 1997).

The functions of plant cuticles can only be understood on the basis of their characteristic wax composition and biosynthetic origin. Plant cuticular waxes are typically mixtures of compounds with one primary (terminal) functional group, i.e. fatty acids, aldehydes, and primary alcohols, as well as esters formed from wax alcohols and fatty acids, or of the corresponding alkanes lacking functional groups (Walton, 1990; Jetter *et al.*, 2006). In some plant species further constituents with mid-chain functional groups occur, for example secondary alcohols and ketones. These can be accompanied by compounds with two secondary functional groups such as diols, ketols, and diketones. All these classes comprise compounds with varying chain lengths, typically ranging from C24 to C32 (Jetter *et al.*, 2006).

The quantitative composition of the mixtures varies greatly between species and sometimes between organs and...
developmental stages (Jetter et al., 2006). Conversely, the wax mixture of a given species (and organ/ontogenetic stage) usually shows only little plasticity, and is characterized by constant relative amounts of compound classes, chain length distributions within these classes, and, for compounds with secondary functional groups, also isomer compositions. This overall chemical profile reflects the characteristics of the biosynthetic machinery and, hence, can ultimately be traced back to the specificities of the involved enzymes as well as their substrate availabilities. Therefore, comparisons between the chain length and isomer profiles of compound classes have been used to infer precursor–product relationships and biosynthetic pathways leading to the various compound classes (Samuels et al., 2008). For example, it was postulated that alkanes serve as precursors for the formation of secondary alcohols, which can be further converted into ketones and finally into ketols (Holloway and Brown, 1977). However, it should be noted that this hypothetical pathway has so far been inferred only from indirect evidence. It has been assembled from comparisons across species, since not all compound classes involved have been reported from a single species.

Plant cuticular waxes rich in alkanes were frequently found to contain large amounts of secondary alcohols together with corresponding ketones (Baker, 1974; Hunt et al., 1976; Holloway et al., 1977; Jenks et al., 1996). In some cases, these were further accompanied by small amounts of bifunctional components such as alkanediols and ketols: *Pisum sativum* leaf wax was reported to contain series of secondary alcohols and alkanes together with alkanediol isomers (Wen et al., 2006). *Brassica oleracea* leaf wax consists mainly of C_{29} alkane (nonacosane), C_{29} secondary alcohols (nonacosan-14-ol and nonacosan-15-ol), and C_{29} ketone (nonacosan-15-one), accompanied by small amounts of C_{29} ketols and diols (Holloway and Brown, 1977; Holloway et al., 1977; Eigenbrode et al., 1991, 1998). Similarly, the stem wax of *Arabidopsis thaliana* is dominated by C_{29} alkanes, secondary alcohols, and ketones (Jenks et al., 1995), and, based on the overall similarity between both Brassicaceae species, it seems likely that *Arabidopsis* wax also contains C_{29} ketols and diols. However, neither of these bifunctional compounds has been reported for this species, even though its leaf and stem waxes have been analysed very thoroughly over the last 15 years.

Parts of the pathway leading from alkanes to ketols have also been substantiated by molecular and biochemical data. There is direct biochemical evidence supporting the biosynthetic relationship between alkanes, secondary alcohols, and ketones for *B. oleracea* (Kolattukudy and Liu, 1970; Kolattukudy et al., 1973). This was recently confirmed by molecular data for *A. thaliana*, where the gene MAH1 encoding a P450-dependent enzyme was cloned and characterized using multiple mutant lines and transgenic plants overexpressing the enzyme in various organs (Greer et al., 2007). Taking the chemical, biochemical, and molecular data together, there is now convincing evidence that alkanes are precursors for secondary alcohols as well as ketones. However, further biosynthetic steps along the postulated pathway leading to diols and ketols have not been confirmed biochemically. It would be possible to study the biosynthesis of these downstream products using *mah1* mutants or MAH1 overexpressors, if these compounds were present in the cuticular wax of *Arabidopsis* wax.

The goal of the present study was to search for bifunctional compounds in the cuticular waxes of *Arabidopsis* wild-type plants. After both diols and ketols had been detected, the chain length and isomer compositions of these compound classes were determined by gas chromatography (GC) and mass spectrometry (MS). The profiles of both compound classes were compared with those of alkanes, secondary alcohols, and ketones in order to assess precursor–product relationships. Finally, the compositions of all wax constituents with secondary functional groups were analysed in *Arabidopsis* mutant and overexpressor lines to test whether the MAH1 enzyme is involved in more than one step along the pathway leading to ketols.

### Materials and methods

#### Plant material

*Arabidopsis thaliana* seeds were spread upon *Arabidopsis* agar plates (Somerville et al., 1982) with 5 mM KNO_{3}, 2.5 mM KH_{2}PO_{4}, 2 mM MgSO_{4}, 2 mM Ca(NO_{3})_{2}, 50 μM NaFe(EDTA), 70 μM H_{2}BO_{3}, 14 μM MnCl_{2}, 10 μM NaCl, 1 μM ZnSO_{4}, 0.2 μM NaMoO_{4}, 0.05 μM CuSO_{4}, 0.01 μM CoCl_{2}, and 7% agar, pH adjusted to 5.6 with KOH, and then stratified for 2–4 d at 4 °C. Plates were placed under continuous light (∼150 μmol m^{-2} s^{-1} photosynthetically active radiation) for 7–10 d at 21 °C for germination. Young seedlings were then transplanted into soil [1:1 ratio of Sunshine Mix 5 (SunGro Horticulture) and Seeding Mix (West Creek Farms)] and grown under the same light and temperature conditions as above.

#### Wax extraction

Leaves or stems were harvested from plants 4–7 weeks after plating. Total cuticular wax mixtures were extracted by immersing whole organs twice for 30 s in chloroform (CHCl_{3}). The two solutions were combined, C_{24} alkane (n-tetracosane) was added as an internal standard, and the solvent was completely evaporated under vacuum.

#### Derivatization reactions

For all the wax analyses, compounds containing free hydroxyl groups were transformed into trimethylsilyl (TMSi) ethers by reaction with bis-N,O-(trimethylsilyl)-trifluoroacetamide (BSTFA) in pyridine for 30 min at 70 °C. To identify ketone isomers and ketols, these two fractions were subjected to reduction by excess lithium aluminium hydride (LiAlH_{4}) in refluxing tetrahydrofuran overnight, hydrolysis with 10% H_{2}SO_{4}, and extraction of the solution with CHCl_{3}.
Identification of secondary alcohols, ketones, alkanediols, and ketols

Total wax mixtures were separated by thin-layer chromatography [TLC; sandwich technique (Tantisawat et al., 1969), silica gel, mobile phase CHCl₃:ethanol 99:1] and resulting fractions were visualized by staining with primuline and UV light. Fractions containing secondary alcohols, ketones, alkanediols, and ketols were removed from the plates, eluted with CHCl₃, filtered, concentrated in a stream of N₂, and stored at 4 °C. After derivatization, constituents were studied with capillary GC (5890N, Agilent, Avondale, PA, USA; column 30 m HP-1, 0.32 mm i.d., df=0.1 µm) with He carrier gas inlet pressure programmed for a constant flow of 1.4 ml min⁻¹ and a mass spectrometric detector (5973N, Agilent). GC was carried out with temperature-programmed on-column injection at 50 °C, oven 2 min at 50 °C, raised by 40°C min⁻¹ to 200 °C, held for 2 min at 200 °C, raised by 3 °C min⁻¹ to 320 °C, and held for 30 min at 320 °C.

Quantification of secondary alcohols, ketones, alkanediols, and ketols

The amounts of secondary alcohols, ketones, and α-ketols were determined by GC with a flame ionization detector (FID) after adding a defined amount of n-tetracosane into the total wax extracts as an internal standard. The GC temperature program was the same as above, but with inlet pressure programmed for constant flow of 2.0 ml min⁻¹ with H₂ as carrier gas. Relative compositions (weight%) of isomers within each compound class were quantified based on the abundance of characteristic MS fragments. The amounts of alkanediols and β-ketols were quantified based on the relative abundance of MS fragment m/z 285 compared with that of α-ketols. The total abundance of fragments m/z 271, 285, and 299 in the β-ketol GC–MS peak was divided by the total abundance of fragments m/z 285 and 299 in the α-ketol peak. Similarly, the amounts of alkanediols were quantified by comparison with the α-ketols using α-fragments m/z 271, 285, and 299. Wax loads (µg cm⁻²) were determined by dividing the amounts of compounds by the surface area extracted for the corresponding sample. Total leaf surface areas were calculated with ImageJ software (Abramoff and Magelhaes, 2004) by measuring the apparent leaf areas in digital photographs and multiplying by two. Stem surface areas were calculated by measuring the projected two-dimensional stem areas in photographs and multiplying by π.

Results

To identify all the homologues and isomers of secondary alcohols and ketones, and to identify alkanediols and ketols, Arabidopsis wild-type stem wax was separated by TLC into five fractions of interest. Two fractions were suspected to contain secondary alcohols (Rᵣ 0.56) and ketones (Rᵣ 0.80) as they co-migrated with the respective standards. The other three fractions were found to contain compounds that had not previously been described for Arabidopsis. The first one, designated as compound class A (Rᵣ 0.65), migrated between secondary and primary alcohols. The second one, designated as compound class B (Rᵣ 0.32), co-migrated with primary alcohols. The third one, designated as compound class C (Rᵣ 0.19), migrated between primary alcohols and fatty acids.

Identification of secondary alcohol and ketone isomers

The TLC fraction co-migrating with secondary alcohols was derivatized with BSTFA and separated by GC, resulting in five peaks with similar mass spectral characteristics. The most dominant GC peak in this fraction was found to contain nonacosan-14-ol and nonacosan-15-ol, two C₂₉ secondary alcohols that had previously been reported for Arabidopsis stem wax. In addition to the fragment ions originating from these two compounds, a pair of α-fragments m/z 271 ([C₁₃H₂₆OTMSi]⁺) and m/z 327 ([C₁₇H₃₄OTMSi]⁺) were detected in the mass spectrum of the same GC peak. Thus, nonacosan-13-ol was identified as a third isomer with the same chain length. The compounds in the other four GC peaks had molecular ions [CₙH₂ₙ₊₁OTMSi]⁺ and corresponding daughter ions [M-CH₃]⁺ that differed by 14 mass units, indicating that these compounds belonged to a homologous series of C₂₇, C₂₈, C₃₀, and C₃₁ secondary alcohols. Pairs of α-ions were used to identify heptacosan-12-ol, -13-ol, and -14-ol, octacosan-13-ol and -14-ol, triactan-14-ol and -15-ol, as well as hentriacontan-14-ol, -15-ol, and -16-ol. Overall, 13 secondary alcohols were identified in this fraction. In Arabidopsis, only nonacosan-14-ol and -15-ol had been identified as individual constituents before, while the C₂₇ and C₃₁ homologues had been reported without specifying isomers, and the C₂₈ and C₃₀ homologues had not been detected. All the 13 secondary alcohols identified here had been described for B. napus and B. oleracea leaf waxes (Holloway et al., 1977).

The TLC fraction co-migrating with ketones was separated by GC into three peaks. Nonacosan-15-one was identified in the major GC peak by the characteristic ketone fragments m/z 225 [CH₃(CH₂)₃CO]⁺ and m/z 241. Pairs of ketone α-ions m/z 211 [CH₃(CH₂)₂CO]⁺ and 239 [CH₃(CH₂)₄CO]⁺ as well as m/z 197 [CH₃(CH₂)₁₁CO]⁺ and 253 [CH₃(CH₂)₁₉CO]⁺ suggested the presence of nonacosan-14-one and nonacosan-13-one. To confirm the isomer composition, the ketone fraction was subjected to reduction with LiAlH₄. The resulting mixture showed a single GC peak containing the C₂₉ secondary alcohols nonacosan-13-ol, -14-ol, and -15-ol. Thus, the original wax fraction must have contained the three corresponding ketones. The compounds in the two minor GC peaks also showed ketone fragmentation patterns. Based on the molecular ions, the compounds in these two peaks were identified as ketones with chain lengths C₂₈ and C₃₀. Because the abundance of the C₂₈ and C₃₀ homologues was very low, the isomer composition of these two homologues could not be determined. In summary, nonacosan-13-one, -14-one, and
-15-one were identified, together with two other minor ketone homologues with chain lengths C_{28} and C_{30}. Nonacosen-15-one was the only compound that had been reported in *Arabidopsis* wax before. In *B. napus* and *B. oleracea* leaf waxes, nonacosan-13-one, -14-one, and -15-one had been detected together with C_{28}, C_{30}, and other homologous ketones. The isomer compositions of the C_{28} and C_{30} ketones had not been resolved (Holloway *et al.*, 1977).

**Identification of ketols and alkanediols**

Compound class A migrated between secondary and primary alcohols on TLC, and therefore probably contained compounds with a secondary hydroxyl group and one (or more) carbonyl group(s). GC analysis of A after BSTFA derivatization resulted in one peak with MS fragments m/z 73, 75, and 103 characteristic for OTMSi groups, but lacking a diol signal m/z 147 [(TMSi)_{2}OH]^+, thus overall indicating the presence of only one hydroxyl group (Fig. 1A, C). Signals at m/z 510 and 495 could be interpreted as a molecular ion and a daughter ion ([M-CH_{3}]^+), respectively. Finally, pairs of ions m/z 225 ([C_{14}H_{29}O]^{+}) and m/z 211 ([C_{14}H_{27}O]^{+}) as well as m/z 299 ([CH_{3}(CH_{2})_{13}CHOTMSi]^{+}) and m/z 285 ([CH_{3}(CH_{2})_{12}CHOTMSi]^{+}) represented α-bond fragments of carbonyl as well as hydroxyl groups, respectively. Taking all this evidence together, two C_{29} α-ketols could be identified within the GC peak as the 15-hydroxynonacosan-14-one and 14-hydroxy-nonacosan-15-one.

Reduction of fraction A with LiAlH_{4} and derivatization by BSTFA yielded a single compound characterized by alcohol fragments m/z 73, 75, and 103, and a diol fragment m/z 147, suggesting the presence of two hydroxyl groups. The alkanediol nature and positions of the functional groups were confirmed by α-fragments m/z 285 ([C_{14}H_{28}OTMSi]^{+}) and m/z 299 ([C_{15}H_{30}OTMSi]^{+}) containing one OTMSi group, and α-fragments m/z 387 ([C_{15}H_{30}OTMSi]^{+}) and m/z 401 ([C_{16}H_{31}OTMSi]^{+}) containing two OTMSi groups.
A molecular ion was detected at \( m/z \) 584 ([C\(_{29}\)H\(_{58}\)(OTMSi)\(_2\)]\(^+\)), accompanied by daughter ions \( m/z \) 569 ([M-CH\(_3\)]\(^+\)) and \( m/z \) 494 ([M-TMSiOH]\(^+\)) (Fig. 1B, C). Taking all this evidence together, the reduced compound was identified as nonacosane-14,15-diol, thus confirming the presence of two functional groups on C-14 and C-15 in all the compounds in fraction A. Therefore, the structures of compounds in fraction A were confirmed as 15-hydroxynonacosan-14-one and 14-hydroxynonacosan-15-one. These structure assignments are in agreement with MS data previously published for the same two \( \alpha \)-ketols in B. napus and B. oleracea leaf waxes (Holloway and Brown, 1977).

GC analysis of fraction B after BSTFA derivatization resulted in one peak with MS fragments at \( m/z \) 73, 75, and 103, indicating the presence of only one hydroxyl group (Fig. 2A). Two series of \( \alpha \)-ions differing by 14 mass units indicated the presence of both a ketone and a secondary alcohol group. This was further supported by the TLC behaviour of the fraction, and by the presence of a fragment \( m/z \) 130 characteristic for \( \beta \)-ketols. The molecular ion \( m/z \) 510 and the daughter ion \( m/z \) 495 ([M-CH\(_3\)]\(^+\)) indicated C\(_{29}\) ketols. Based on pairwise combinations of \( \alpha \)-fragments, 16-hydroxynonacosan-14-one, 15-hydroxynonacosan-13-one, and 13-hydroxynonacosan-15-one were then identified.

To confirm the tentative structures, fraction B was reduced by LiAlH\(_4\) and derivatized by BSTFA. The MS data allowed the identification of nonacosane-14,16-diol and nonacosane-13,15-diol. Therefore, the previously unknown compounds in this fraction of Arabidopsis stem wax were identified as 16-hydroxynonacosan-14-one, 15-hydroxynonacosan-13-one, and 13-hydroxynonacosan-15-one. All three structures were finally corroborated by comparison with published MS data for the same \( \beta \)-ketols in B. napus and B. oleracea leaf waxes (Holloway and Brown, 1977).

GC analysis of fraction C after BSTFA derivatization resulted in one peak with MS characteristics similar to those of the mixture of nonacosane-14,16-diol and nonacosane-13,15-diol TMSi derivatives (Fig. 3). However, the relative

![Fig. 2. Mass spectra and fragmentation diagram of secondary/secondary \( \beta \)-ketols in the stem wax of Arabidopsis. (A) Mass spectrum of the bis TMSi ether of \( \beta \)-ketol isomers, (B) mass spectrum of the bis TMSi ether of secondary/secondary \( \beta \)-alkanediol isomers, and (C) mass spectral fragmentation diagram of TMSi derivatives of \( \beta \)-ketols and \( \beta \)-alkanediols.](https://academic.oup.com/jxb/article-abstract/60/6/1811/518700)
abundance of the α-fragment m/z 299 was much higher than that of m/z 271, suggesting that this fraction must have contained at least one other nonacosanediol isomer. Because only three α-ions were detected in the mass spectrum, the α-ion m/z 299 could only originate from an isomer that also gave the signals at m/z 271 or 285. Thus, nonacosane-14,15-diol was the only possible structure that could account for the high relative abundance of m/z 299. To confirm that there were no carbonyl groups, fraction C was subjected to LiAlH₄ reduction. The mass spectral data of the resulting mixture were the same as those before LiAlH₄ treatment (data not shown). Thus, fraction C was found to contain nonacosane-14,15-diol, nonacosane-14,16-diol, and nonacosane-13,15-diol. The latter two alkanediols were identified for the first time in nature.

Quantification of mid-chain functionalized compounds in the stem wax of wild-type Arabidopsis

Secondary alcohols and ketones in the waxes of wild-type Arabidopsis stems, mah1-1 mutant stems, and MAH1-over-expressing leaves were quantified (coverage, µg cm⁻²) by GC with an FID. The isomers within each homologue could not be separated by GC because differences in the geometry of functional groups between the multiple isomers were too small. Consequently, the relative amounts (%) of isomers were also quantified by the relative abundance of characteristic MS α-ions.

Arabidopsis wild-type stem wax contained 9.5% (2.4 µg cm⁻²) of secondary alcohols. The C29 homologue accounted for 98% of the fraction, while only trace amounts of the other homologues between C27 and C31 could be detected. Each of the homologues with even-numbered chain lengths was dominated by one isomer. For example, the C28 and C30 secondary alcohols were dominated by octacosan-14-ol (91%) and triacontan-15-ol (93%), respectively (Table 1). In contrast, the isomers were more evenly distributed within the homologues with odd-numbered chain lengths. The C29 homologue was composed of nonacosan-14-ol and -15-ol in a ratio of 1:2. The C27 homologue contained heptacosan-12-ol, -13-ol, and -14-ol in a ratio of ~1:4:5, while the C31 homologue contained equal amounts of hentriacontan-15-ol and -16-ol.

Ketones were the second dominant compound class, accumulating to 24% (6.0 µg cm⁻²) of the total stem wax. The C29 homologue accounted for >99% of this compound class. Only trace amounts of C28 and C30 homologues were detected. The C29 homologue contained 94% of nonacosan-15-one and 6% of nonacosan-14-one. Due to the extremely low amounts of the C28 and C30 homologues, their isomer compositions could not be quantified (Table 1).

The ketols were minor components in Arabidopsis wild-type stem wax. The coverage of the α-ketols was 0.16 µg cm⁻² (0.1% of the total stem wax mixture). Due to their low abundance, the coverage of the β-ketols could not be quantified by GC–FID. The β-ketols were thus estimated as 4% of the α-ketols by the abundance of their MS α-ions. Within the α-ketols, 14-hydroxynonacosan-15-one and 15-hydroxynonacosan-14-one accounted for 73% and 27%, respectively (Table 1). Within the β-ketols, 16-hydroxynonacosan-14-one accounted for 80% while 15-hydroxynonacosan-13-one and 13-hydroxynonacosan-15-one amounted to 9% and 11%, respectively (Table 1).

Since the alkanediols co-eluted with triacontanal in GC analysis of total wax mixtures, the coverage of the alkanediols could not be quantified by GC–FID. However, the amounts of alkanediols could be assessed using a procedure similar to that used for quantification of the β-ketols, and were found to be present at ~33% of the α-ketol level (0.05 µg cm⁻²). Nonacosane-14,15-diol was the most abundant isomer (61%) of all the alkanediols. The other two isomers, nonacosen-14,16-diol and nonacosen-13,15-diol, contributed 31% and 8%, respectively (Table 1).

Quantification of mid-chain functionalized compounds in the stem wax of mah1 mutants

It had been described that the secondary alcohols in the knockout mutant mah1-1 stem wax were decreased to 1% of the wild-type level (Greer et al., 2007), thus, mah1-1 provided a convenient tool to study the biosynthetic relationship between secondary alcohols and ketols. In the following, the stem wax of mah1-1 was further analysed to quantify the residual compounds containing secondary functional groups.

Only very small amounts of secondary alcohols were detected in the mah1-1 stem wax (0.1 µg cm⁻²). The isomer composition of the mutant secondary alcohols was very similar to that of the wild-type stem wax. All the secondary alcohols detected in wild-type stem wax were found in the mutant, except for triacontan-14-ol (Table 2). Octacosan-14-ol (92%) and triacontan-15-ol (99%) strongly dominated the...

Fig. 3. Mass spectrum of the bis TMSi ether of secondary/secondary alkanediols in the stem wax of Arabidopsis.
Table 1. Relative homologue and isomer compositions (%) of secondary alcohols, ketones, secondary/secondary alkanediols, ketols, and alkanes in the total wax of wild-type Arabidopsis stems

| Homologue chain length | Homologue composition | Isomer composition within homologues | Homologue chain length | Homologue composition |
|------------------------|-----------------------|-------------------------------------|------------------------|-----------------------|
|                        |                       | C-12  | C-13  | C-14  | C-15  | C-16  |                        |                       | C-12  | C-13  | C-14  | C-15  | C-16  |                        |
| Secondary Alcohols     |                       |       |       |       |       |       |                        |                       |       |       |       |       |       |                        |
| C27                    | tr^2                 | 8.5±0.9 | 39.3±0.3 | 52.1±0.4 |       |       |                        |                       |       |       |       |       |       |                        |
| C28                    | tr                    | 9.7±0.5 | 90.7±0.2 |       |       |       |                        |                       |       |       |       |       |       |                        |
| C29                    | 98.4                  | 1.2±0.02 | 36.4±0.7 | 62.4±0.9 |       |       |                        |                       |       |       |       |       |       |                        |
| C30^2                  | tr                    | 7.3    | 92.7   |       |       |       |                        |                       |       |       |       |       |       |                        |
| C31^2                  | tr                    | 4.2    | 49.1   | 46.7  |       |       |                        |                       |       |       |       |       |       |                        |
| Ketones                |                       |       |       |       |       |       |                        |                       |       |       |       |       |       |                        |
| C28                    |                       | 0.2±0.01 | 5.7±1.2 | 94.1±0.3 |       |       |                        |                       |       |       |       |       |       |                        |
| C29                    |                       |       |       |       |       |       |                        |                       |       |       |       |       |       |                        |
| C30                    |                       |       |       |       |       |       |                        |                       |       |       |       |       |       |                        |
| Alkanediols^6          |                       |       |       |       |       |       |                        |                       |       |       |       |       |       |                        |
| C29                    | 100                   | C-13,15 | C-14,16 | C-14,15 |       |       |                        |                       |       |       |       |       |       |                        |
| α-Ketols^5              |                       |       |       |       |       |       |                        |                       |       |       |       |       |       |                        |
| C29                    | 100                   | C-14^4 | C-15^4 | C-14^4 |       |       |                        |                       |       |       |       |       |       |                        |
| β-Ketols^6              |                       |       |       |       |       |       |                        |                       |       |       |       |       |       |                        |
| C29                    | 100                   | C-13^4 | C-15^4 | C-14^4 |       |       |                        |                       |       |       |       |       |       |                        |

1 The values are given as mean (n=5) ± SE unless otherwise specified. Homologues were quantified within compound classes, and isomers were quantified within each chain length.
2 tr: <0.5%.
3 The isomer composition was calculated based on two GC-runs of the TLC-separated samples, thus the SE is not shown.
4 Positions of carbonyl groups.
5 The isomer compositions were calculated by the relative abundance of α-ions.
6 The isomer composition was calculated by averaging three sets of α-ions from one GC-MS run of the TLC-separated sample.

Table 2. Relative homologue and isomer compositions (%) of secondary alcohols, ketones, and alkanes in the total stem wax of the Arabidopsis mutant mah1-1

| Homologue chain length | Homologue composition | Isomer composition within homologues^2 | Homologue chain length | Homologue composition |
|------------------------|-----------------------|---------------------------------------|------------------------|-----------------------|
|                        |                       | C-12  | C-13  | C-14  | C-15  | C-16  |                        |                       | C-12  | C-13  | C-14  | C-15  | C-16  |                        |
| Secondary Alcohols     |                       |       |       |       |       |       |                        |                       |       |       |       |       |       |                        |
| C27                    | tr^3                 | 16    | 27    | 57    |       |       |                        |                       |       |       |       |       |       |                        |
| C28                    | tr                    | 8     | 92    |       |       |       |                        |                       |       |       |       |       |       |                        |
| C29                    | 99                    | 1     | 37    | 61    |       |       |                        |                       |       |       |       |       |       |                        |
| C30                    | tr                    | 1     | 99    |       |       |       |                        |                       |       |       |       |       |       |                        |
| C31                    | tr                    | 4     | 47    | 49    |       |       |                        |                       |       |       |       |       |       |                        |
| Ketones                |                       |       |       |       |       |       |                        |                       |       |       |       |       |       |                        |
| C29                    | 100                   | 3     | 97    |       |       |       |                        |                       |       |       |       |       |       |                        |

1 The homologue and isomer compositions were calculated based on one GC-MS run of the TLC-separated sample.
2 Values are given as mean (n=5) ± SE.
3 tr: trace, <0.5%.

C28 and C30 homologues, respectively. Heptacosan-12-ol, -13-ol, and -14-ol were found in a ratio of ~2:3:6. The C29 homologue contained mainly nonacosan-14-ol and -15-ol in a ratio of 1:2. The two major isomers within the C31 homologue, hentriacontan-15-ol and -16-ol, had similar abundance (48% each). In addition to secondary alcohols, trace amounts of C29 ketones were detected in mah1-I stem wax. The C29 ketones were found to be composed of 97% nonacosan-15-one and 3% nonacosan-14-one (Table 2). Alkanediols and ketols were not detected in mah1-I stem wax.

Quantification of mid-chain functionalized compounds in the leaf wax of MAH1-overexpressing mah1 mutants

The leaf wax of Arabidopsis wild type contains a large percentage of alkanes but only trace amounts of secondary
alcohols (Jenks et al., 1995). Therefore, leaves provide a convenient system to study the biochemical properties of MAH1. Plants ectopically overexpressing MAH1 under the control of the cauliflower mosaic virus (CaMV) 35S promoter had previously been generated in the mah1-1 background (Greer et al., 2007). In the present study, the leaf waxes from two individual lines (a and b) of these transgenic plants were analysed. They were found to contain relatively high levels of secondary alcohols and ketones that could be quantified by GC–FID and GC–MS (Table 3). C29 and C31 secondary alcohols were detected in the leaf waxes of both transgenic lines. The secondary alcohols were composed of nonacosan-12-ol, -13-ol, -14-ol, and -15-ol, as well as hentriacontan-13-ol, -14-ol, -15-ol, and -16-ol. Within the C29 homologue, nonacosan-15-ol and nonacosan-14-ol were found in a 2:1 ratio, while the other two isomers were present at much lower levels (1.6% each). Hentriacontan-15-ol and -16-ol had similar amounts (47% each) within the C31 homologue. In contrast to the strong dominance of the C29 homologue (98%) in wild-type stem wax, the C29 homologue accounted for only 54% of all the secondary alcohols in the leaf wax of the transgenic Arabidopsis. The secondary alcohols amounted to 4.2% and 3.2% of the total leaf waxes in each of the transgenic lines, respectively. In addition to secondary alcohols, ketones were also detected and found to contain 4% nonacosan-14-one and 96% nonacosan-15-one. Ketones amounted to 3.7% and 3.1% of the total leaf waxes of the two individual lines. No alkanediols and ketols were detected in the wax of the transgenic leaves.

Discussion

In the present study, Arabidopsis wax components containing secondary functional groups were examined (i) to test the biosynthetic relationship between secondary alcohols and ketols and (ii) to determine the regiospecificity and substrate preference of the enzyme involved in ketol biosynthesis and, thus, to test the possible involvement of MAH1 in ketol biosynthesis. Both the precursor–product relationships and the specificity of MAH1 can now be discussed based on the data presented here.

Table 3. Relative homologue and isomer compositions (%) of secondary alcohols, ketones, and alkanes in the total leaf wax of the MAH1-overexpressing lines (a and b)

Homologues were quantified within compound classes, and isomers were quantified within each chain length.

| Homologue chain length | Homologue composition | Isomer composition within homologues | Homologue chain length | Homologue composition |
|------------------------|-----------------------|--------------------------------------|------------------------|-----------------------|
|                        |                       | C-12       | C-13       | C-14       | C-15       | C-16       |                       |                       |
|                        | a         | b         | a         | b         | a         | b         | a         | b         | a         | b         |
| Secondary Alcohols     |                       |           |           |           |           |           |           |           |           |           |
| C29                    | 55.1      | 53.5      | 1.6       | 1.8       | 1.6       | 1.4       | 33.3      | 31.7      | 63.5      | 65.1      |
| C31                    | 44.9      | 46.5      | 2.8       | 3.1       | 2.7       | 3.3       | 49.3      | 51.7      | 45.2      | 42.8      |
| Ketones                | 100       | 100       | 5.7       | 6.2       | 94.3      | 93.8      |           |           |           |           |
| Alkanes                |                       |           |           |           |           |           |           |           |           |           |
| C29                    | 33.6      | 32.0      |           |           |           |           |           |           |           |           |
| C31                    | 66.4      | 68.0      |           |           |           |           |           |           |           |           |

Conversion of alkanes to secondary alcohols by MAH1: substrate chain length and product isomer preferences

In vitro assays with radioactively labelled precursors had shown that alkanes are the substrates for secondary alcohol biosynthesis in B. oleracea (Kolattukudy and Liu, 1970). By analogy, it can be assumed that alkanes also serve as substrates for formation of the same secondary alcohols in Arabidopsis. The alkane and secondary alcohol fractions of Arabidopsis stem wax have matching chain length distributions (dominated by C29), and the alcohol profile may thus be accounted for by alkane substrate availability alone. Therefore, the substrate specificity of the hydroxylase involved cannot be assessed based on the wild-type wax composition alone. It is possible that this hydroxylase either accepts a relatively broad substrate spectrum, or it has a preference for C29 alkane.

It had been demonstrated that MAH1 is the enzyme responsible for hydroxylating alkanes into secondary alcohols in Arabidopsis (Greer et al., 2007). In the current study, transgenic Arabidopsis leaves overexpressing MAH1 were found to contain C29 and C31 alkanes in a ratio of 1:2, while the homologue ratio of the corresponding secondary alcohol products was 6:5. This result clearly shows that the product composition can differ from that of the substrate, indicating that MAH1 has a preference for the C29 alkane. However, the product ratio found for the overexpressor leaves also differed from that in wild-type stems, showing that the different substrate pools available in both systems also affected product formation. Thus, both the substrate preference of MAH1 and the substrate availability together determine the homologue distribution of secondary alcohols in Arabidopsis wax.

The secondary alcohols in Arabidopsis wild-type stem wax had been reported to contain multiple isomers (Rashotte et al., 2001, 2004), suggesting that MAH1 hydroxylates on more than one carbon position. To determine the range of hydroxylated carbon atoms, the previously unknown secondary alcohol isomers were identified in the current study. In each of the five secondary alcohol homologues (C27 to C31), only two or three isomers were detected, demonstrating that MAH1 hydroxylates on a narrow range of carbons. Based on the relative isomer abundances, it can be inferred that the
enzyme preferentially hydroxylates: (i) the central carbon (e.g. C-13 in C_{27} alkane, C-14 in C_{28} alkane, C-15 in C_{29} alkane) and/or (ii) on C-15 (e.g. leading to nonacosan-15-ol and hentriacontan-15-ol).

**Conversion of secondary alcohols to ketones by MAH1: substrate chain length and product isomer preferences**

Indirect evidence had previously indicated that MAH1 can use secondary alcohols as substrates for a second hydroxylation on the carbon carrying the hydroxyl group, leading to a geminal diol structure that will rearrange into a carbonyl function (Greer *et al.*, 2007). The strong dominance of nonacosan-15-one indicated that MAH1 has the same regioselectivity in the second hydroxylation as that in the first hydroxylation. The substrate chain length preference for the second hydroxylation step of MAH1 is also consistent with that for the first hydroxylation step. In the leaf wax of MAH1 overexpressors, the C_{29} and C_{31} secondary alcohols were detected at a similar abundance, while only C_{29} ketones were found, demonstrating that MAH1 prefers to hydroxylate C_{29} secondary alcohols.

**Substrate–product relationships between secondary alcohols, ketones, alkanediols, and ketols**

Ketols had been described for the leaf waxes of *B. napus* and *B. oleracea* (Holloway and Brown, 1977). Because the positions of functional groups in these ketols are very similar to those of the co-occurring secondary alcohols and ketones, it had been proposed that secondary alcohols and ketones serve as the substrates for ketol biosynthesis in the Brassicas (Holloway and Brown, 1977). In the current study, ketols were identified for the first time in wild-type *Arabidopsis* stem wax. Interestingly, it was found that the functional groups of these ketols were located on carbon positions similar to those of the hydroxyl groups in the accompanying secondary alcohols. This correlation provided further indirect evidence to support the precursor-product relationship between secondary alcohols and the bifunctional constituents, here in a second plant genus beside the Brassicas. As a third line of evidence, the absence of the bifunctional components from the stem wax of the *Arabidopsis mah1* mutant also confirmed the substrate-precursor relationship between secondary alcohols and ketols. Taking all these findings together, it can now be concluded that ketols are formed as derivatives of secondary alcohols (Fig. 4).

The *Arabidopsis* ketol compositions were closely paralleled by the alkanediol profiles in the same wax mixtures. The wild-type stem wax diols were found to have homologue and isomer compositions similar to those of the secondary alcohols and ketols. The *mah1* mutant stem wax lacked diols and ketols, coincident with reduced levels of secondary alcohols. Based on these results, it is very likely that alkanediols are formed by hydroxylation of secondary alcohol substrates, and that the diols in turn serve as substrates for ketol formation. Thus, it is concluded that alkanediols are intermediates on the pathway from secondary alcohols to ketols (Fig. 4).

Ketones could also serve as intermediates between secondary alcohols and ketols (Fig. 4). The results showed that the isomer and homologue distributions of the ketols matched those of the ketones to a certain degree. This confirms the hypothesis that ketones are intermediates of ketol formation, on pathways parallel to those via alkanediols. However, it should be noted that the quantitative results revealed discrepancies between isomer patterns of ketones and ketols, indicating that the pathways leading via

Fig. 4. Proposed biosynthetic relationships between alkanes, secondary alcohols, ketones, and ketols found in the stem wax of *Arabidopsis*. All the reaction steps may be catalysed by MAH1. The shades of grey in the arrows indicate the regioselectivity of MAH1 (black, hydroxylation on C-15; grey, hydroxylation on C-14; light grey, hydroxylation on C-13).
ketones to ketols account for only a small portion of the ketols formed.

**Conversion of secondary alcohols and ketones to alkanediols and ketols: substrate chain length and product isomer preferences**

All the reaction steps leading from secondary alcohols via ketones or alkanediols to ketols can be explained by simple hydroxylations. They involve either hydroxylation of methylene carbons to secondary hydroxyl groups, or of -CHOH- groups to geminal diols that will rearrange to carbonyl groups. Thus, it is expected that all the transformations occurring on these pathways are catalysed by hydroxylases. Based on the isomer compositional data, the substrate and regiospecificity of the hydroxylases can now be inferred.

All the alkanediols and ketols detected in *Arabidopsis* stems had their functional groups on carbon positions between C-13 and C-15, which was in the same range as the hydroxyl positions in the accompanying secondary alcohols. Furthermore, the most abundant isomers of alkanediols or ketols had one functional group on C-15. Thus, it seems that the hydroxylase(s) involved in alkanediol and ketol biosynthesis has (have) a similar, limited regiospecificity to MAH1. In the stem wax of *Arabidopsis* wild type, only alkanediols and ketols with chain length C$_{29}$ were detected. This result could be due to substrate chain length preference of the hydroxylase(s) or to substrate availability, because the secondary alcohols and ketone substrates for formation of alkanediols and ketols are dominated by C$_{29}$ homologues.

In conclusion, MAH1 and the hydroxylase(s) involved in alkanediol/ketol biosynthesis have similar regiospecificity and may also have the same substrate chain length preference. This raises the possibility that all the *Arabidopsis* wax components with secondary functional groups are formed by the same enzyme, MAH1, through single, double, or triple hydroxylation of alkanes. However, the different reaction steps differ in the numbers of hydroxyl groups that are present in the substrates handled in each step. Thus, if MAH1 is indeed the only enzyme involved, then it must be relatively flexible with respect to the functional groups present on or near the carbon it hydroxylates. This hypothesis is plausible since MAH1 also exhibits only limited regiospecificity, implying promiscuity not only for the exact substrate configuration but also for product geometry.

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