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EPICUTICULAR WAX VARIATION IN ECOTYPES OF *ARABIDOPSIS THALIANA*

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Key Word Index—*Arabidopsis thaliana*; Cruciferae; ecotypes; epicuticular wax; primary alcohols.

Abstract—Quantification of the epicuticular wax from the stems of 40 ecotypes of *Arabidopsis thaliana* showed a two-fold range in total wax load that was not correlated to known abiotic characteristics of the ecotype's origin of collection. Chemical analysis of these ecotypes revealed similar epicuticular wax profiles for all ecotypes except CT-1. In CT-1 the amount of 22 and 24 carbon length primary alcohols was increased by 16- and 8-fold, respectively, over that observed in the epicuticular wax averaged over all ecotypes. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Epicuticular wax forms an above ground boundary layer between a plant and its environment. This epicuticular wax layer, found on the surface of all plants, is primarily composed of seven major chemical classes in dicotyledonous plants: aldehydes, alkanes, esters, free fatty acids, ketones, and primary and secondary alcohols [1, 2]. The mixture of these chemical components, known as the epicuticular wax profile, has been found to be very similar within a specific taxonomic classification of plants and has been used as a character in taxonomic keys [3, 4].

In *Arabidopsis thaliana* a variety of mutants with altered epicuticular wax profiles have been studied, however, all of these mutants have been generated in one of two ecotypic backgrounds [5, 6]. Initial observations indicated that the epicuticular wax profiles of these two ecotypes were very similar except for the total wax load [7]. An ecotype is defined as a population in a specific environment that has maintained its identity through isolation or selection [8].

Our chemical analysis of an ecotype was concentrated on the stem tissue. This was done: (1) to capitalize on the wax load which is 10–20 times higher on the stems than the leaves [7]; (2) to reduce the variation caused by potential differences in epicuticular wax load on the abaxial and adaxial leaf surfaces of various ecotypes; and (3) because of our ongoing interest in aphid herbivory on *Arabidopsis* in which stems is the primary feeding site (Rashotte and Feldmann, unpublished).

The analysis of the epicuticular wax from these 40 ecotypes was conducted in a search for additional genetic variation that might help unravel the biosynthetic and secretory pathways of epicuticular waxes. The analysis was also done to determine how much overall variation existed in the epicuticular waxes and how that might provide valuable data for studies on plant–insect interactions.

RESULTS AND DISCUSSION

Epicuticular wax load per area sampled was examined in 40 ecotypes of *A. thaliana* that originated from vastly different worldwide environments. There was a two-fold variation in total stem epicuticular wax load between the different ecotypes, ranging from just over 1500 to almost 3000 $\mu g\ dm^{-2}$ (Table 1). One might expect epicuticular wax load to be correlated with environmental conditions of the ecotype's site of origin, since differing amounts of UV-light, acid rain, humidity, temperature and insect attack, among other factors are known to affect wax load [6, 9–12]. When grown under similar environmental conditions, we found no correlation between an ecotype's epicuticular wax load and the known values of altitude, daily temperature, monthly rainfall, the longitude and the latitude for the ecotype's original site of collection. However, there are other environmental factors not documented for ecotype origin, which may directly correlate to epicuticular wax load. We are currently

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Table 1. Epicuticular wax load of *A. thaliana* ecotypes. Ecotype abbreviation, origin of collection and accession number are as documented from the Nottingham and Ohio State Resource Centers

Ecotype	Origin of collection	Accession number	EW load ± s.e.*
Cnt-1	Canterbury, U.K.	1635	1531 ± 31 a†
Hi-0	Hilversum, Netherlands	1226	1596 ± 41 ab
Lc-0	Loch Ness, U.K.	1306	1632 ± 98 ab
Sch	Schwanheim, Germany	—	1653 ± 28 b
En-T	Enkheim/Frankfurt, Germany	921	1674 ± 83 ab
Per-1	Perm, Russia	1444	1695 ± 170 ab
Ws-2	Wassilewskija, Russia	1601	1702 ± 80 ab
Ca-0	Camberg/Taunus, Germany	1060	1737 ± 89 ab
Hl-0	Holtensen, Germany	1228	1746 ± 55 b
Col-0	Missouri, U.S.A.	1092	1773 ± 27 b
Pog-0	British Columbia, Canada	1476	1786 ± 30 b
Shahdara	Pamiro-Alay, Tadjikistan	929	1791 ± 129 bc
Ler-1	Landsberg, Germany	1642	1854 ± 73 bc
Rd-0	Rodenbach Dill, Germany	1482	1856 ± 183 bc
Bay-0	Bayreth, Germany	954	1873 ± 72 bc
Tsu-0	Tsu, Japan	1564	1896 ± 61 bc
Est-1	Estland, Estonia	1152	1900 ± 118 bc
Rld-1	Rschew, Russia	913	1903 ± 54 bc
Mt-0	Martuba/Cyrenaika, Libya	1380	1951 ± 63 c
Hm	Hannover-Munden, Germany	—	1953 ± 17 c
Wi	Wisselheim, Germany		2013 ± 81 c
An-1	Antwerpen, Belgium	944	2047 ± 106 cd
Tu-0	Turin, Italy	1566	2047 ± 49 c
Li-0	Limberg, Germany	1310	2081 ± 60 c
Dijon-M	Dijon, France	919	2083 ± 46 c
Mh-0	Muhlen, Poland	1366	2117 ± 104 cd
Sei-0	Seis am Schlern, Italy	1504	2182 ± 105 cd
Oy-0	Oystese, Norway	1436	2199 ± 110 cd
Wl-0	Wildbad, Germany	1630	2200 ± 147 cd
Lip-0	Lipowiec/Chrzanow, Poland	1336	2226 ± 73 cd
Kin-0	Missouri, U.S.A.	1272	2236 ± 105 cd
Jl-1	Vranov, Czechoslovakia	1248	2271 ± 65 d
Bs-1	Basel, Switzerland	996	2294 ± 62 d
Co-1	Coimbra, Portugal	1084	2315 ± 148 cd
Cvi-0	Cape Verde Islands	1096	2342 ± 81 d
Ct-1	Catania, Sicily	1094	2419 ± 95 d
In-0	Innsbruck, Austria	1238	2443 ± 95 d
Ll-0	Llagostera, Spain	1338	2600 ± 230 d
Sap-0	Slapy, Czechoslovakia	1506	2668 ± 171 d
HOG‡	Khurmatou, Tadjikistan	922	2953 ± 153 d

*Epicuticular wax (EW) load is presented as an average of at least three replicates and is measured in $\mu g\ dm^{-2}$ ± s.e.

†Every letter indicates significant difference (t-test, $\alpha = 0.05$) from other letters; values with two letters are not significantly different from either letter group.

‡Unabbreviated name Hodja-Obi-Garm.

involved in laboratory studies to address insect selection pressure as an additional factor influencing wax load.

Examination of the stem epicuticular wax of these ecotypes showed their overall profile of chemical constituents to be similar. A correlation test comparing ecotypes, using all the chemical components in each ecotype's wax profile, never yielded a correlation coefficient value below 0.98 for any two ecotypes (data not shown), indicating that there are virtually no differences in stem epicuticular wax profiles, except for load, between ecotypes. This high level of similarity between ecotypes allowed us to generate a representative stem epicuticular wax profile for *A. thaliana* (Fig. 1) averaged over all replicates of every ecotype sampled. Even the ecotypes with the lowest and highest epicuticular wax loads, CNT-1 and HOG, respectively, conform very closely in chain length distribution of constituents to the average wax profile (Fig. 1). This similarity is somewhat unexpected in light of other characters studied among ecotypes, such as pathogen resistance, carbon dioxide responses and water use efficiency, which have been shown to vary considerably [8, 13–15]. It seems that there is a set epicuticular wax profile for *A. thaliana* under the conditions used in this experiment. However, it is possible

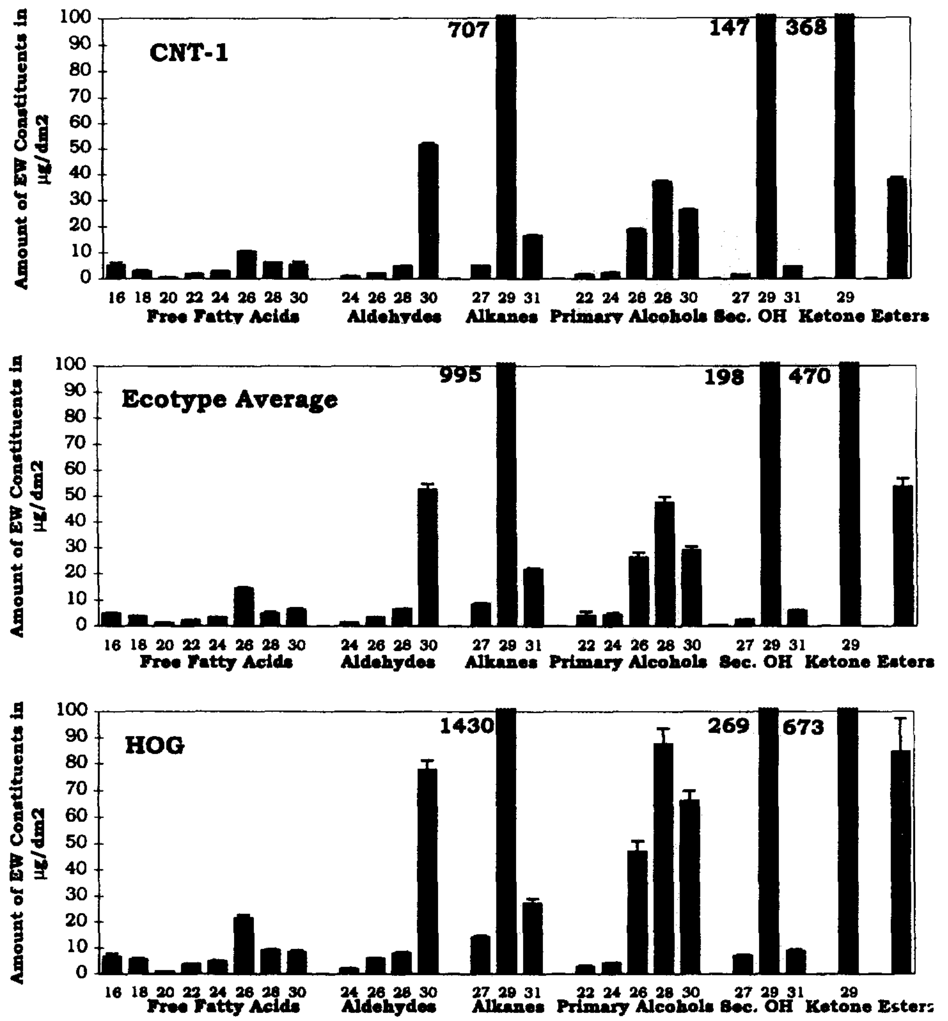

Fig. 1. Epicuticular wax profiles of *A. thaliana* ecotypes. Each bar shows the amount of a specific epicuticular wax constituent, labeled on the *x*-axis by an epicuticular wax class and a carbon unit length of that constituent (Sec OH is the abbreviation for secondary alcohols). All measurements are in μg dm^{-2} ± s.e. Bars that reach the top of the graph have additional numbers to the left of them, indicating the actual epicuticular wax amount, which is off the *y*-axis. Top: the profile of the CNT-1 ecotype (this ecotype had the lowest wax load of those sampled). Middle: this is the ecotype average, which was calculated by averaging the individual averages of all replicates for each ecotype. Bottom: the profile of the HOG ecotype (this ecotype had the highest wax load of those sampled).

that in the original environments where these ecotypes were collected, epicuticular wax profiles may vary due to temperature, light, humidity and other environmental effects.

A more detailed examination of the individual chemical classes in these ecotypes yielded one with a different and novel EW profile: CT-1. In CT-1 the chain length distribution of the primary alcohol class differs significantly from all other ecotypes (Fig. 2). The C_{22} and C_{24} primary alcohols of CT-1 are 16- and 8-fold higher, respectively, than the amounts observed in the average ecotype, while the C_{26}, C_{28}, and C_{30} primary alcohols of CT-1 and the average ecotype are present in very similar amounts. This profile is also unique among epicuticular wax mutants in Arabidopsis [5, 6], and all other plant species sampled to date. It is not clear what has occurred to cause this increase in C_{22} and C_{24} primary alcohols. It is possible that there has been a shift in the flow of constituents though the epicuticular wax biosynthetic pathway. For example, since wax esters are formed from the esterification of free fatty acids and primary alcohols in the wax there could be a reduced utilization of C_{22} and C_{24} primary alcohols in ester biosynthesis. In fact, preliminary data show the ester profile of CT-1 is different than the other ecotypes (data not shown).

The abiotic environmental factors known about the origin of collection, show no unusual specific factor or combination of factors that might indicate the cause for the increase of C_{22} and C_{24} primary alcohols in CT-1. Still, it is possible that other abiotic or biotic environmental pressures unknown about the origin of collection may have caused this change. One possibility is that the wax profile change occurred in

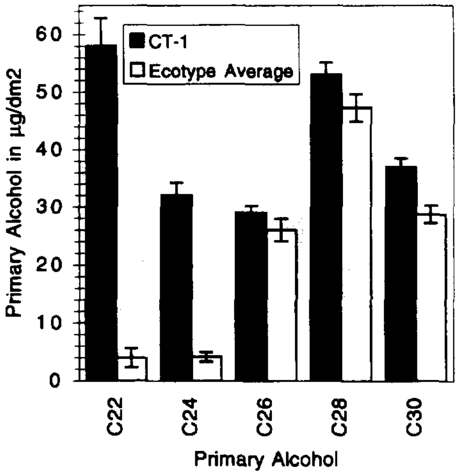

Fig. 2. The primary alcohol chain length distributions for the ecotype average and CT-1. The ecotype average profile was calculated by averaging all replicates for every ecotype. The CT-1 primary alcohol profile was generated from the average of three individual replicates. C_{22}, C_{24}, C_{26}, C_{28} and C_{30} in the legend refer to the carbon unit length of each primary alcohol. All amounts are measured in $\mu g\ dm^{-2} \pm$ s.e.

response to high insect selection pressure, since both a high alcohol/aldehyde ratio and shorter chain length wax constituents in epicuticular wax have been implicated in insect resistance [16]. Not only does the CT-1 profile change match both these implicated areas, but CT-1 has shown a reduced level of aphid fecundity in comparisons to other ecotypes with wax profiles similar to the ecotype average in Fig. 1 (Rashotte and Feldmann, unpublished).

In conclusion, we have sampled the epicuticular waxes from 40 ecotypes of *A. thaliana* originating from vastly different environments worldwide, and we were able to present a highly conserved stem epicuticular wax constituent profile for this species. Epicuticular wax loads examined from these ecotypes had a two-fold range in total load. Novel variation in the epicuticular wax profile was discovered in the ecotype CT-1. The increase in short chain primary alcohols in CT-1 is unique among species sampled, and studies are underway to determine whether these differences may affect insect herbivory.

EXPERIMENTAL

Plant material. We employed 40 different ecotypes of *A. thaliana* which were obtained from the Nottingham and Ohio State Arabidopsis Resource Centers; see Table 1 for names and accession numbers. Also available from the Resource Centers were environmental factors known about the origin of collection. All plants were grown in a controlled environment chamber at 22° for 16 hr in the light (240 μmol $m^{-2} sec^{-1}$; 75% RH) and 18° for 8 hr in the dark (75% RH).

Epicuticular wax analysis. The hexane soluble surface lipids (designated epicuticular wax) were extracted from stems of 25–30-day-old plants by immersing tissues in hexane for 30 sec. Immediately, before extracting the wax, the stems were photocopied. Exact area measurements were made later by tracing the photocopied image of each stem using a digitizer mouse and the computer program Sigma Scan. At least three separate replicates of stem tissue were used in calculating an average epicuticular wax profile for each ecotype and all replicates from every ecotype sampled were used in calculating the ecotype average epicuticular wax profile (Fig. 1). Derivatization, gas chromatography, mass spectrometry and quantification of identified epicuticular wax constituents based on correction factors developed using one int. standard and calibration curves of 18 external standards were applied as performed according to ref. [17].

Chemicals identified as epicuticular wax constituents on A. thaliana. Hexadecanoic, octadecanoic, eicosanoic, docosanoic, tetracosanoic, hexacosanoic, octacosanoic, triacontanoic acids are described here as C_{16}, C_{18}, C_{20}, C_{22}, C_{24}, C_{26}, C_{28}, and C_{30} free fatty acids; tetracosanal, hexacosanal, octacosanal, triacontanal as C_{24}, C_{26}, C_{28}, and C_{30} aldehydes; *n*-heptacosane, *n*-nonacosane, and *n*-hentriacosane as C_{27}, C_{29}, and C_{31} alkanes; 1-docosanol, 1-tetracosanol, 1-hexacosanol, 1-octacosanol, and 1-triacontanol, as C_{22}, C_{24}, C_{26}, C_{28} and C_{30} primary alcohols; 13- and 14-heptacosanol, 14- and 15-nonacosanol, 15- and 16-hentriacontanol as C_{27}, C_{29}, and C_{31} secondary alcohols; and 15-nonacosanone as C_{29} ketone.

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