Chapter 8

METABOLOMICS OF CUTICULAR WAXES: A SYSTEM FOR METABOLOMICS ANALYSIS OF A SINGLE TISSUE-TYPE IN A MULTICELLULAR ORGANISM

M. Ann D.N. Perera and Basil J. Nikolau
W.M. Keck Metabolomics Research Laboratory, Iowa State University, Ames, IA 50011, USA

Abstract: Electrospray ionization tandem mass spectrometry in the precursor and neutral loss scanning modes is utilized to obtain profiles of the complex, polar lipids of plants. This method is rapid, accurate, and sensitive. The technique is being used to determine the metabolic functions of known genes, to implicate new metabolic pathways, and to help identify mutant genes from their functions.

Key Words: lipidomics; lipid profiling; electrospray ionization; mass spectrometry; phospholipids; galactolipids.

1 INTRODUCTION

The emerging field of metabolomics seeks to globally identify the low molecular weight (<1,000 Da) biochemical constituents of biological materials (Hall et al., 2002; Bino et al., 2004). These molecules are primarily either metabolites of intermediary metabolism or end products of metabolism. These molecules therefore represent the final level at which most genes express their functionality (Fiehn et al., 2000; Fiehn, 2002; Weckwerth and Fiehn, 2002). Hence, one of the many potential utilities of metabolomics data is in the field of functional genomics, which seeks to identify the biochemical and physiological function of all genes in a genome (Weckwerth and Fiehn, 2002; Bino et al., 2004). The application of metabolomics data to functional genomics faces a number of inherent limitations. One of these is the fact that many individual metabolites are common to different metabolic processes.

B.J. Nikolau and E. Syrkin Wurtele (eds.), Concepts in Plant Metabolomics, 111–123.

Thus, unlike proteomics and transcriptomics, where there is a one-to-one correspondence between individual molecules (proteins and mRNAs) and individual genes, no such correlation exists for individual metabolite molecules (Oliver et al., 2002). Another limitation of metabolomics, at least as currently practiced for eukaryotic multicellular organisms, is the lack of discrimination of metabolites from different cellular and subcellular compartments. Namely, because metabolomic analyses are usually conducted on metabolite extracts made by the homogenization of a number of different tissue types and subcellular compartments, data concerning the spatial arrangement of metabolites is lost. This is of particular significance in the case of metabolites that are common to different metabolic processes that occur in distinct cellular and subcellular compartments (e.g., acetyl-CoA; (Ke et al., 2000; Fatland et al., 2005)).

Cuticular waxes are constituents of the cuticle that coat all aerial organs of terrestrial plants (Martin and Juniper, 1970; Post-Beittenmiller, 1996; Kunst and Samuels, 2003). Because cuticular waxes are products of the metabolism of a single cell layer of plants (i.e., the epidermis), their metabolomic analysis offers a convenient system for evaluating the utility of metabolomics in functional genomics in the absence of the complexity associated with cellular compartmentalization of metabolites. Furthermore, because cuticular waxes are extracellular and they are not covalently bound to the organism, they are a readily extracted and analyzed. Thus, the "exometabolome" of the aerial organs of terrestrial plants has the potential of assessing the metabolic status of the epidermis.

The biological function of the cuticle is complex and not precisely defined (Martin and Juniper, 1970). It has been implicated as having a role in plant–water relationships, and in responses of plants to biotic and abiotic stimuli. Although biochemical studies have provided the skeleton of the metabolic processes that underlie the biosynthesis of this lipid mixture, the genetic and molecular regulation of this process is poorly understood (Post-Beittenmiller, 1996; Kunst and Samuels, 2003). A number of mutant collections that affect the normal accumulation of cuticular waxes are available for elucidating the molecular genetics of cuticular wax biosynthesis. These include an extensive mutant collection in Arabidopsis (the cer mutants; (Jenks et al., 1995; Jenks et al., 1996), in barley (the cer-que mutants; von-Weittstein-Knowles, 1986), and in maize (the glossy mutants; (Schnable et al., 1994)). Additional, but less extensive collections have also been generated in cabbage (Eigenbrode et al., 1995), pea (Macey and Barber, 1970), and sorghum (Jenks et al., 2000). Each of these collections offers unique opportunities for combined biochemical and genetic studies that should reveal different aspects of a very complex metabolic process. This chapter presents the procedures that have been developed for the metabolomics analysis of the cuticular waxes of maize and Arabidopsis.

2 MATERIALS AND METHODS

2.1 Plant materials

The maize (*Zea mays*) inbred line B73 was used in all the studies presented herein. Seeds were germinated in a sand-bench maintained in a greenhouse whose temperature was maintained at 25°C. Plants were illuminated for 16 hours per day with natural sunlight, supplemented with artificial lighting, at a level of 210 μmol m^{-2} s^{-1}. Seedlings at the 2-leaf stage (7-9 days old) were harvested at between 4- and 6-hours after the start of the illumination period, by cutting seedlings at ground level. The coleoptile was removed from the harvested seedling and cuticular waxes were immediately extracted.

The Columbia ecotype of Arabidopsis thaliana was used in all the studies reported herein. Seeds were germinated in soil (professional growing mix Sun Gro LC1) and plants were grown in a growth-chamber, which was maintained at constant illumination level of 60-80 μmol m^{-2} s^{-1}, at a temperature of 22°C, and 75% relative humidity. Waxes were extracted from the bolt, when it was approximately 15 cm tall.

2.2 Extraction of cuticular waxes

The harvested plant material was transiently immersed in chloroform for 60 seconds. The chloroform extract was filtered through a plug of glass wool and the filtrate was dried under reduced pressure in a rotary evaporator at 30°C. The dried wax sample was dissolved in a small volume (250 mL) of chloroform and analyzed *via* HPLC or GC-MS.

2.3 HPLC separation of cuticular waxes

Cuticular wax extracts were separated into their chemical classes by reverse phase HPLC. Chromatography was conducted with a 53 mm x 7 mm (3 μm particle size) Adsorbosphere C18 (12% C) column (Altech, Deerfiled, IL), using a Beckman 110B HPLC system. The flow rate was at 1.0 mL/min. Elution was monitored with an evaporative light scattering detector (ELSD 11A, Varex, Maryland). The nebulizer and the evaporator of the detector were set at 70°C. For maize cuticular waxes the HPLC solvent gradient system was: 0-10 min, 100% THF; 10-20 min, linear gradient to heptane:THF (70:30); 20-25 min at THF:heptane (70:30); 25-36 min, 100% heptane; 36-40 min, 100% THF. For Arabidopsis cuticular waxes the HPLC solvent gradient system was: 0–7 min, 100% THF; 7–17 min, linear gradient to THF:heptane:hexane) (70:15:15); 17–25 min, pentane:hexane (50:50), 25–31 min, linear gradient to 100% THF. Fractions containing constituents of

different chemical classes (i.e., alkanes, alkenes, alcohols, aldehydes, esters, fatty acids, and ketones) were collected using a fraction collector. All chemical standards used in these studies were purchased from Altech (Deerfield, IL, USA).

2.4 Gas chromatography-mass spectrometric analysis

Chromatographic analysis was performed with a Model 6890 series gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a Model 5973 mass detector (Agilent Technologies, Palo Alto, CA, USA). Chromatography was conducted with a 30 m length, 0.25 mm I.D. HP-5MS cross-linked (5%)-diphenyl-(95%)-dimethyl polysiloxane column, using helium as the carrier gas. The injection temperature was at 300°C. The column oven temperature was initially at 80°C and was increased to 260°C at a rate of 5°C/min. After holding this temperature for 10 minutes, it was ramped to 320°C at a rate of 5°C/min and held at this temperature for 30 min, and finally cooled to the starting temperature (80°C) over a 5-minute interval. Using the HP enhanced chemical analysis software G1701BA version B.01.00 with Windows NT^{TM} operating system facilitated peak identification.

2.5 Analysis of unsaturated metabolites

The position of carbon–carbon (C–C) double bonds in unsaturated components was determined by the GC-MS analysis of dimethyl disulfide adducts. Isolated cuticular waxes (~1 mg) were dissolved in 20 mL of heptane, and incubated overnight at 40°C°C with 50 mL dimethyl disulfide, and 5 mL 0.06% (w/v) I_2 in diethyl ether. The reaction was stopped by the addition 50 mL heptane, and 25 mL aqueous solution of (5% w/v) sodium thiosulfate. The organic phase was recovered and concentrated prior to GC-MS analysis.

3 RESULTS AND DISCUSSION

To facilitate the complete identification of cuticular wax constituents, extracted cuticular waxes were separated to chemical class components *via* HPLC. Figure 8-1A shows the fractionation of maize waxes into the five major chemical class constituents (aldehydes, alcohols, ketones, esters, and alkanes), and Figure 8-1B shows the similar fractionation of Arabidopsis waxes. The identity of each peak was based on the co-elution with known standard mixtures for each chemical class. These standards were n-alkanes

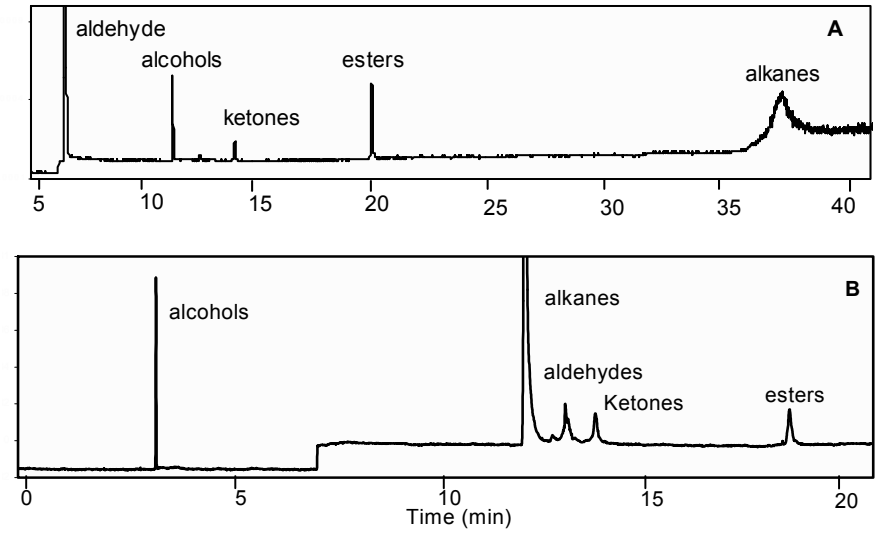

Figure 8-1. The HPLC fractionation of cuticular waxes. Cuticular waxes were extracted from 9-day-old maize seedling leaves (A) and rosette leaves of 21-days old Arabidopsis seedling (B), and fractionated by reverse phase HPLC coupled to an evaporative light scattering detector.

(between 12- and 26-carbons), alcohols (1-octacosanol, 1-octadecanol and 1-docosanol), ketones (2-heptadecanone, 14-heptacosanone, and 6-tricosanone), esters (docosanyl eicosanoate, docosanyl docosanoate, and docosanyl hexacosanoate), aldehydes (octadecanal, decanal, and dodecanal) and n-fatty acid acids (mixture of 16–20 carbon chain lengths).

The fractionated chemical classes were collected, and each fraction was then analyzed *via* GC-MS. By comparing the total ion chromatographic (TIC) profile of the unfractionated cuticular wax extract with that of the TIC of each fraction, it was possible to classify each individual cuticular wax constituent to a chemical class. Thus, this HPLC pre-fractionation, simplified the identification of the cuticular wax constituents prior to GC-MS analysis. Figures 8-2A–C illustrates the application of this strategy for identifying the alcohol and aldehyde constituents of maize cuticular waxes. These analyses identified aldehydes of between 16 and 32 carbon chain lengths (Figure 8-2A), and alcohols of similar chain length distribution (Figure 8-2C). Figure 8-3 illustrates the identification of the cuticular wax components isolated from bolts and siliques of Arabidopsis.

Verification of the chemical identity of each metabolite was achieved by the interpretation of the mass spectra obtained from the electron-impact (EI) ionization/fragmentation of each metabolite. The interpretation of these mass-spectra is illustrated with an example of a metabolite for each chemical class. Figure 8-4A presents the mass spectrum of the 29-carbon n-alkane.

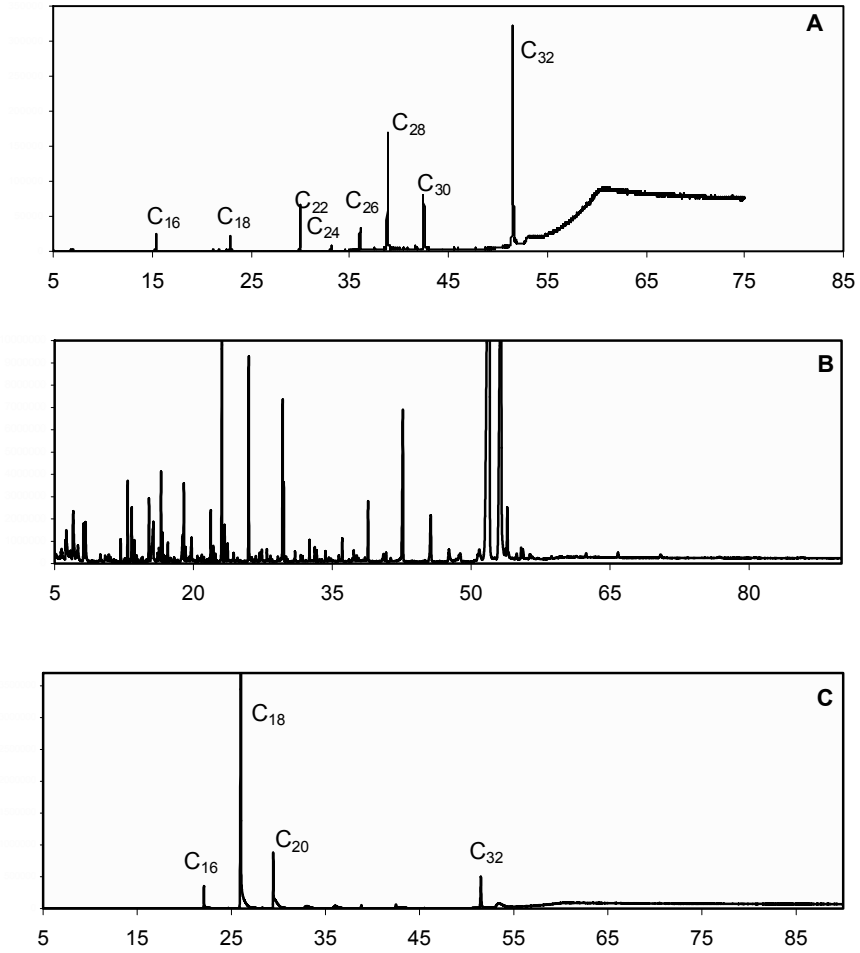

Figure 8-2. Identification of cuticular wax components by combined HPLC and GC fractionation. Isolated maize cuticular waxes were separated into an alcohol and aldehyde fractions by HPLC. The purified alcohol (A) and aldehyde fractions (C) were analyzed by GC, and the resultant chromatograms are compared to the chromatograms of the isolated cuticular waxes (B).

Typical of linear hydrocarbons the spectrum is composed of clusters of fragmentation products that differ from each other by 14 *m/z* mass units, which represents loss of $(CH_2)_nCH_3$ groups from the molecular ion. The *m/z* value of the molecular ion (408 units), which confirms the identity of this alkane as an alkane of 29-carbons. Primary alcohols are identified by the signature loss of a water molecule from the molecular ion, which leads to the increased abundance, relative to that of the molecular ion, of an ion of 18

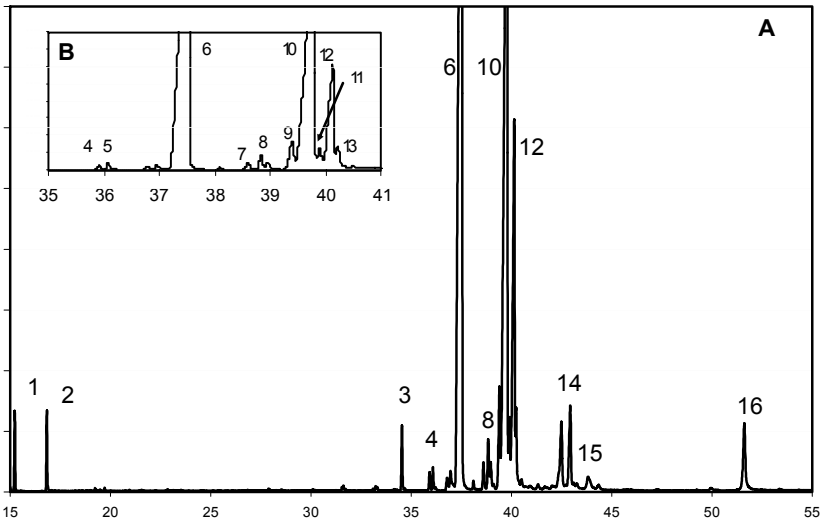

Figure 8-3. GC chromatography of Arabidopsis cuticular waxes. Insert B, is an expanded view of the chromatogram between 35 and 41 minutes of elution time. Peaks were identified as: 1, hexadecanoic acid; 2,octadecanoic acid; 3, 1-tetracosanol; 4, heptacosane; 5, 13-heptacosanone; 6, nonacosane; 7, hexacosanoic acid; 8, secondary alcohol of hexacosanol; 9, 1-octacosanol; 10, 15-nonacosanone; 11, octacosanal; 12, 1- triacontanol; 13, hentriacosane; 14, triacontanal; 15, amyrin; 16, C_{44} ester.

m/z units less than the molecular ion. Thus, as illustrated in Figure 8-4B, the molecular ion of 466 *m/z* units, in combination with the water-loss fragment of 448 *m/z* units, identifies this metabolite as a primary alcohol of 32 carbon chain length.

The signature base-ion facilitates the mass-spectroscopic identification of aldehydes (Figure 8-4C). This fragmentation ion is due to the loss of a CHO group resulting in an ion that is 29 *m/z* units smaller than the molecular ion. However, identification of aldehydes based solely on such a fragmentation pattern is complicated by the fact that alkanes also generate such a fragmentation pattern by the loss of a CH_2–CH_3 group. This complication was clarified by the fact that we had pre-fractionated the alkanes and aldehydes *via* HPLC (Figures 8-1A and B), and thus could independently identify these two classes of metabolites.

Esters are the only molecules present in cuticular waxes that are "hybrid" molecules, being composed of an alcohol and acid moieties. Thus, their characterization required the identification of both moieties. Depending on the combination of these two moieties, each ester at a defined carbon chain length could consist of several isomers, i.e., C_{44} esters could be isomers of C_{20} acid + C_{24} alcohol, C_{22} acid + C_{22} alcohol, C_{24} acid + C_{20} alcohol etc. Our strategy of pre-fractionating the cuticular wax extract *via* HPLC,

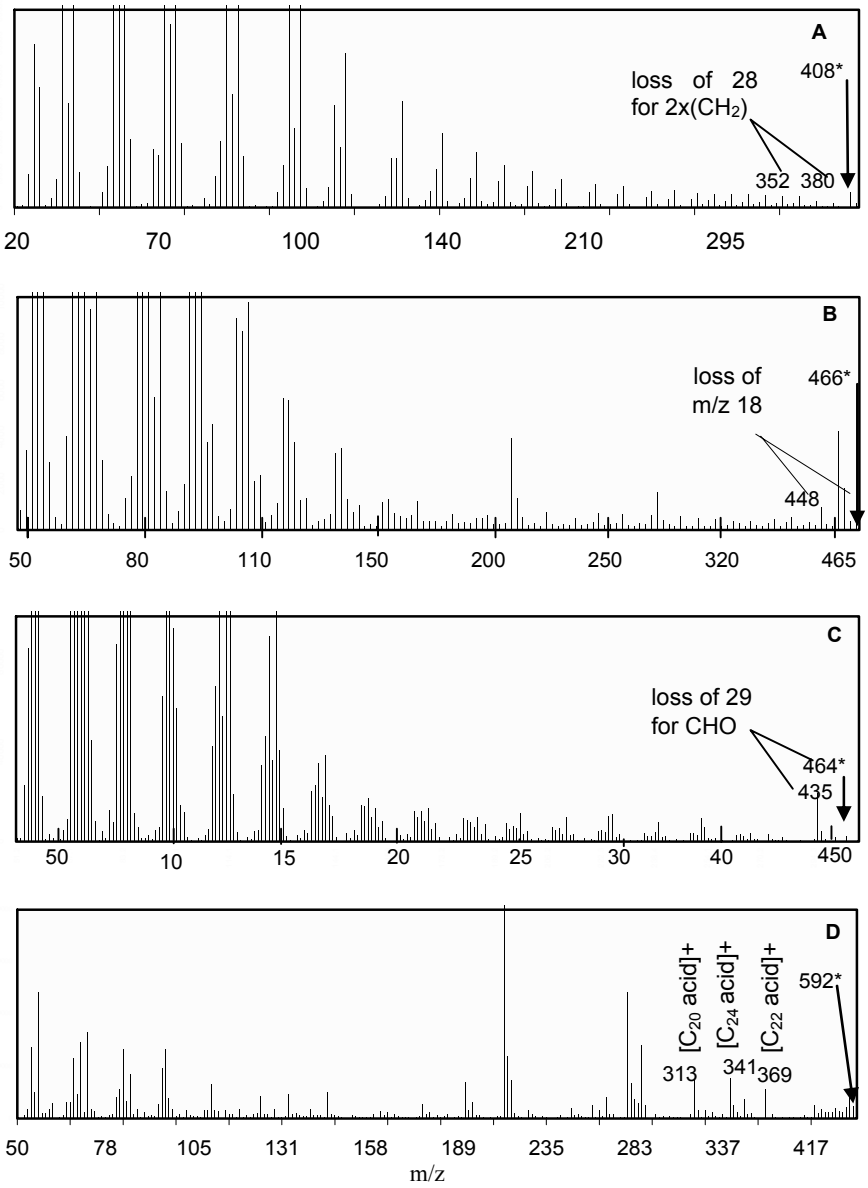

Figure 8-4. Mass-spectra of cuticular wax components. Characteristics of the mass spectra that lead to the identification of alkanes (A), alcohols (B), aldehydes (C) and esters (D) are illustrated with nonacosane, dotriacontanol, dotriacontanal, and C40 ester, respectively.

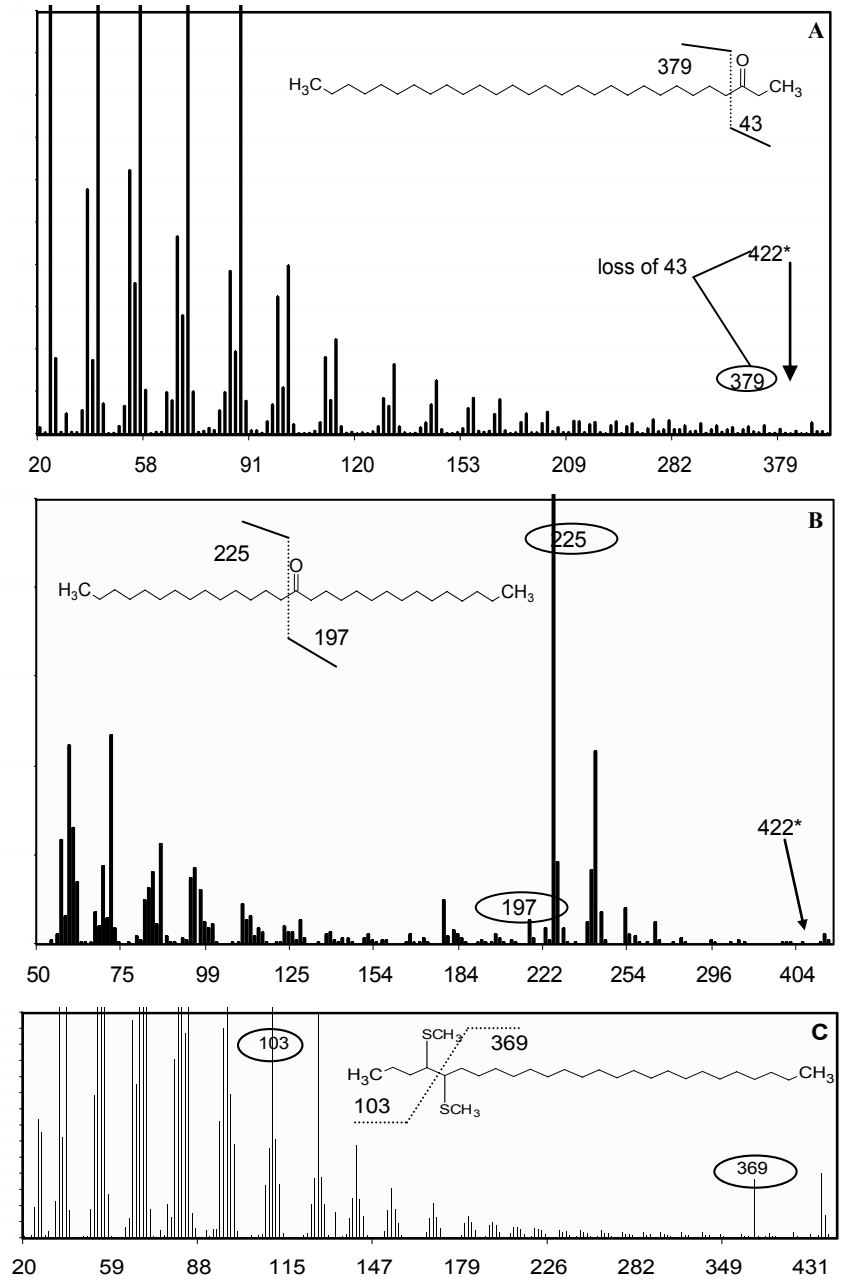

Figure 8-5. Mass-spectra of cuticular wax components. Characteristics of the mass spectra that lead to the identification of methylketones (A), symmetrical ketone (B), and alkenes (C) are illustrated with 2-nonacosanone, 15-nonacosanone, and dimethyl disulfide adduct of 4-heptacosene, respectively.

enabled us to identify these metabolites, however, the fractionation of these esters *via* GC fractionates these metabolites only on the basis of their total carbon number. Ester isomers with the same total carbon number, but differing in their acid and alcohol moieties were identified *via* the characteristic protonated acid fragmentation ions (Reiter et al., 1999). Figure 8-4D, illustrates such an analysis of the C40 ester (identified as such by the *m/z* value of the molecular ion), which is a mixture of three isomers that are each composed of C_{20} acid + C_{20} alcohol, C_{22} acid + C_{18} alcohol, and C_{24} acid + C_{16} alcohol.

Once the identity of the ketones was established by the HPLC fractionation, GC-MS analyses identified the carbon chain length of these molecules and the position of the carbonyl group. The former could be calculated from the *m/z* value of the molecular ion; for example, Figure 8-5 illustrates two isomers of 29-carbon ketones, both of which display a molecular ion of 422 *m/z* units. However, these isomers display distinct fragmentation patterns that reveal the different position of the carbonyl group. EI-induced fragmentation generates ions from the cleavage of the C–C bonds adjacent to the carbonyl group. Thus, in maize the carbonyl group is at the C-2 position (i.e., they are methyl ketones) generating a characteristic ion that is 43 *m/z* units less than the molecular ion due to the loss of a $CO–CH_3$ group (Figure 8-5A). In contrast, the Arabidopsis ketones have a centrally located carbonyl group, which fragment to generate stable base-ions as illustrated in Figure 8-5B. Thus, the Arabidopsis cuticular waxes contain symmetric ketones.

The cuticular waxes of some maize organs, particularly silk and pollen contain unsaturated components. These were identified as alkenes, dienes, aldehydes, and methyl ketones. The position(s) of the carbon-carbon double bonds on these metabolites was identified by the GC-MS analysis of dimethyl disulfide adducts. Upon MS analysis, such adducts preferentially fragment at the bond between the carbon atoms that have been derivatized, yielding two substantial fragment ions that identify the positions of the carbon atoms involved in the carbon-carbon double bond. Figure 8-5C, illustrates the mass spectrum of the dimethyl disulfide adduct of 4-heptacosene (a 27-carbon alkene, with the double bond at the 4^{th} position), which upon fragmentation generates substantial fragment ions of 103 and 369 *m/z* units.

In total these analyses identified 232 metabolites from the cuticular waxes of maize and Arabidopsis (Table 8-1). Associated with each metabolite is a high-quality mass-spectrum, measured on a double-focusing sector field spectrometer (70 eV EI). In addition, each metabolite is identified *via* retention indices from a nonpolar stationary phase column (HP5, Agilent Technologies, Palo Alto, CA, USA). The resulting database has been useful in the characterization of plants that carry cuticular wax mutations (Nikolau et al., 2002, ; Nikolau et al., 2003; Dietrich et al., 2005;

Perera et al., 2005). Moreover, this database has been used to discover new genes in new biosynthetic pathways (Perera et al., 2005).

Table 8-1. Cuticular wax constituents

Chemical class	Carbon chain lengths[a]	
	Maize	Arabidopsis
Saturated aldehydes	C_{22}, C_{24}, C_{26}, C_{28}, C_{30}, **C_{32}**	C_{26}, C_{28}, **C_{30}**
Unsaturated aldehydes	C_{26}, **C_{28}**, C_{30}	none
Primary alcohols	C_{16}, C_{18}, C_{20}, C_{24}, C_{26}, C_{30}, **C_{32}**	C_{24}, **C_{28}**
Secondary alcohols	none	C_{27}, **C_{29}**
Alkanes	C_{15}, C_{19}, C_{23}, C_{29}, **C_{31}**	C_{27}, **C_{29}**, C_{31}
Alkenes	C_{19}, C_{23}, C_{25}, C_{27}, **C_{29}**, C_{31}	none
Dienes	C_{25}, C_{27}, **C_{29}**, C_{31}	none
Methyl ketones	C_{17}, C_{23}, C_{25}, C_{27}, **C_{31}**	C_{25}, C_{27}, **C_{29}**
Symmetric ketones	none	C_{25}, C_{27}, **C_{29}**
Unsaturated ketones	C_{21}, C_{25}, C_{27}, **C_{29}**, C_{31}	none
Esters	C_{40}, C_{42}, C_{44}, C_{46}, C_{48}, **C_{52}**	C_{42}, **C_{44}**
Esterified alcohols	C_{16}, C_{18}, C_{20}, C_{24}, **C_{26}**, C_{28}, C_{30}, C_{32}	C_{16}, C_{18}, **C_{20}**
Esterified acids	C_{16}, C_{18}, C_{20}, **C_{24}**, C_{26}	C_{18}, C_{20}, **C_{24}**
Free fatty acids	Trace	**C_{16}**, C_{18}, C_{24}, C_{30}

[a]In each chemical class, the carbon chain length of the most abundant metabolite is identified in bold-text.

ACKNOWLEDGEMENTS

This research was supported in part by grants from the National Science Foundation (IBN-9808559 and IOB-0344852) and by Hatch Act and State of Iowa funds.

REFERENCES

Bino, R.J., Hall, R.D., Fiehn, O., Kopka, J., Saito, K., Draper, J., Nikolau, B.J., Mendes, P., Roessner-Tunali, U., Beale, M.H., Trethewey, R.N., Lange, B.M., Wurtele, E.S., and Sumner, L.W., 2004, Potential of metabolomics as a functional genomics tool, *Trends Plant Sci.* **9**:418–425.

Dietrich, C.R., Perera, M.A., M. DY-N, M., Meeley, R.B., Nikolau, B.J., and Schnable, P.S., 2005, Characterization of two GL8 paralogs reveals that the 3-ketoacyl reductase component of fatty acid elongase is essential for maize (Zea mays L.) development, *Plant* **42**:844–861.

Eigenbrode, S.D., Moodie, S., Castagnola, T., 1995, Predators mediate host plant resistance to a phytophagous pest in cabbage with glossy leaf wax. Entomologia Experimentalis et Applicata **77**: 335–342.

Fatland, B., Nikolau, B.J., and Wurtele, E.S., 2005, Reverse Genetic Characterization of Cytosolic Acetyl-CoA Generation by ATP-citrate lyase in Arabidopsis, *Plant Cell* **17**:182–203.

Fiehn, O., 2002 Metabolomics – the link between genotypes and phenotypes, *Plant Mol. Biol.* **48**:155–171.

Fiehn, O., Kopka, J., Dormann, P., Altmann, T., Trethewey, R.N., and Willmitzer, L., 2000, Metabolite profiling for plant functional genomics, *Nat. Biotechnol.* **18**:1157–1161.

Hall, R., Beale, M., Fiehn, O., Hardy, N., Sumner, L., and Bino, R., 2002, Plant metabolomics: the missing link in functional genomics strategies, *Plant Cell* **14**:1437–1440.

Jenks, M.A., Rashotte, A.M., Tuttle, H.A., and Feldmann, K.A., 1996, Mutants in Arabidopsis thaliana Altered in Epicuticular Wax and Leaf Morphology, *Plant Physiol.* **110**:377–385.

Jenks, M.A., Rich, P.J., Rhodes, D., Ashwort, E.N., Axtell, J.D., and Din, C.K., 2000, Leaf sheath cuticular waxes on bloomless and sparse-bloom mutants of Sorghum bicolor, *Phytochemistry* **54**:577–584.

Jenks, M.A., Tuttle, H.A., Eigenbrode, S.D., and Feldmann, K.A., 1995, Leaf Epicuticular Waxes of the Eceriferum Mutants in Arabidopsis, *Plant Physiol* **108**:369–377.

Ke, J., Wen, T.N., Nikolau, B.J., and Wurtele, E.S., 2000, Coordinate regulation of the nuclear and plastidic genes coding for the subunits of the heteromeric acetyl-coenzyme A carboxylase, *Plant Physiol.* **122**:1057–1071.

Kunst, L. and Samuels, A.L., 2003, Biosynthesis and secretion of plant cuticular wax, *Prog. Lipid Res.* **42**:51–80.

Macey, M.J.K. and Barber, H.N., 1970, Chemical genetics of wax formation on leaves of Pisum sativum-D, *Phytochemistry* 9.

Martin, J.T., and Juniper, B.E., 1970, *The Cuticle of Plants*, Edward Arnold Ltd, Edinburgh.

Nikolau, B.J., Perera, M.A., Dietrich, C.R., and Schnable, P.S., 2002, High-throughput metabolomic analysis of an extensive new collection of maize glossy mutants and of

maize inbreds reveals novel genetic regulation of epicticular wax biosynthesis, *In*: 1st International Congress on Plant Metabolomics, Wageningen, The Netherlands.

Nikolau, B.J., Perera, M.A.D.N., Dietrich, C.R., and Schanble, P.S., 2003, Metabolomic analysis of epicuticular wax biosynthesis in maize using a collection of maize glossy mutants, *In*: 2nd International Congress on Plant Metabolomics, Potsdam, Germany.

Oliver, D.J., Nikolau, B., and Wurtele, E.S., 2002, Functional genomics: high-throughput mRNA, protein, and metabolite analyses, *Metab. Eng*. **4**:98–106.

Perera, A., Dietrich, C.R., Schanble, P.S., and Nikolau, B.J., 2005, Metabolomic analysis of a collection of maize glossy mutants reveals novel aspects of cuticular wax biosynthesis, In preparation.

Post-Beittenmiller, D., 1996, Biochemistry and molecular biology of wax production in plants, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**:405–430.

Reiter, B., Marion, L., Lorbeer, E., Aichholz, R., 1999, Isolation and characterization of wax esters in fennel and caraway seed oils by SPE-GC, *J. High Resol.Chromatogr.* **22**:514–520.

Schnable, P.S., Stinard, P.S., Wen, T.-J., Heinen, S., Weber, D., Zhang, L., Hansen, J.D., and Nikolau, B.J., 1994, The genetics of cuticular wax biosynthesis, *Maydica* **39**:279–287.

Von Wettstein-Knowles, P., 1986, Role of *cer-cqu* in epicuticular wax biosynthesis, *Biochemical Society Transactions* **14**:576–579.

Weckwerth, W., and Fiehn, O., 2002, Can we discover novel pathways using metabolomic analysis? *Curr. Opin. Biotechnol.* **13**:156–160.