Composition of cuticular wax on the leaves of *Kalanchoe daigremontiana*

by

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Abstract

Analysis of cuticular wax from *Kalanchoe daigremontiana* leaves was performed to identify the constituent components within the wax, determine how these changed during leaf ontogenesis, and discover how they were distributed within the cuticle.

Analysis of extracted cuticular wax by gas chromatography, mass spectrometry, and comparison with authentic standards led to the identification of triterpenoids including glutinol, friedelin, germanicol, epifriedelanol, glutinol acetate and β-amyrin as well as very long chain fatty acid (VLCFA) derivatives including alkanes, primary alcohols, aldehydes, fatty acids, and alkyl esters. Cuticular wax composition in young *K. daigremontiana* leaves was dominated by triterpenoids, which made up over 70% of the lipid soluble compounds. During leaf ontogenesis, wax composition changed to include a higher proportion of VLCFA derivatives, which made up approximately 50% of cuticular wax in mature leaves. The most abundant triterpenoids in the wax were glutinol and friedelin, both fairly uncommon pentacyclic triterpenoids with a complex proposed biosynthetic mechanism. Tritriacontane (C_{33} alkane) was the most abundant compound within the VLCFA derivatives. Cuticular wax accumulation was found to correspond well to leaf growth, with both processes slowing at the same time. Variations in the ratio of friedelin-like compounds to glutinol-like compounds during leaf ontogenesis suggest the presence more than one active triterpenoid synthase enzyme in the leaves of *K. daigremontiana*.

VLCFA compounds were found mainly in the epicuticular wax on both the adaxial and abaxial surfaces, while triterpenoids were relatively more abundant in the intracuticular layer. Two different epicuticular wax crystal forms were observed by scanning electron microscopy (SEM) which can be described as platelets with sinuate margins and twisted ribbons. Based on SEM and chemical data as well as previous reports of crystal composition, it is hypothesized that each crystal type has a unique composition, with the platelets containing one or more triterpenoids and the twisted ribbons containing alkanes and other VLCFA derivatives. Confirmation of this hypothesis will have to await further investigation.

This research provides information that will aid in the larger goals of characterizing a glutinol or friedelin synthase and understanding the gradients established within epicuticular and intracuticular wax layers.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACP – acyl carrier protein

BSTFA – N,O-Bis (Trimethylsilyl) trifluoroacetamide

cDNA – complementary Deoxyribonucleic Acid

CoA – co-enzyme A

DMAPP – dimethylallyl diphosphate

DOXP/MEP - 1-deoxy-D-xylulose-5-phosphate / 2-C-methyl-D-erythritol 4 –phosphate

FAE – fatty acid elongation complex

FAS – fatty acid synthesis

FPP – farnesyl diphosphate

GC-FID – gas chromatograph(y) - flame ionization detector

GC-MS – gas chromatograph(y) – mass spectrometry

GFP – green fluorescent protein

GPP – geranyl diphosphate

GUS - β-glucuronidase

HPLC – high performance liquid chromatography

IPP – isopentenyl diphosphate

mRNA – messenger ribonucleic acid

NMR – nuclear magnetic resonance

OSC – oxidosqualene cyclase

pcr – polymerase chain reaction

RT-PCR – reverse transcription polymerase chain reaction

SEM – scanning electron microscop(e)/(y)

TLC – thin layer chromatography

TMS – trimethylsilyl

VLCFA – very long chain fatty acid

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Dedication

This thesis is dedicated to Carl. Thank you for everything!

Co-authorship statement

Chapter 2 – Cuticular wax composition in *Kalanchoe daigremontiana* during leaf ontogenesis

Identification and design of research program – I worked from established protocols within the research group to establish specific procedures related to this project. Identification of overall project goals was undertaken in consultation with Dr. Jetter

Performing the research – all data referenced in this chapter, except where indicated, was obtained in experiments performed by myself.

Data analyses – I performed all data analyses for this chapter

Manuscript preparation – I wrote ~80-90% of the text in this chapter, with editing and suggestions from Dr. Jetter. For the version submitted for publication, my contribution was slightly less

Chapter 3 – Epicuticular and Intracuticular wax composition on Kalanchoe daigremontiana leaves

Identification and design of research program – Working from established protocols, I designed experiments to test specific parameters for this project. Project goals were established in consultation with Dr. Jetter.

Performing the research – 90% of the data referenced in this chapter was obtained in experiments performed by myself

Data analyses – I performed all data analyses for this chapter

Manuscript preparation – I wrote 90% of the text in this chapter, with editing and suggestions from Dr. Jetter. For the version that will be submitted for publication, my contribution was about 50-60%

Chapter 1: Introduction

1: Introduction – The Cuticle

Most aerial portions of terrestrial plants are covered by a thin layer known as the cuticle. The primary, although not only, role of the cuticle is to prevent non-stomatal water loss through the epidermis. The cuticle is also the surface through which the plant interacts with the outside world and it has ecological functions as well as its primary physiological one. Chemical and biological approaches have yielded important information about the structure, composition, and function of the cuticle and some of its component parts.

1.1: Cuticle Structure and general composition

The cuticle is a hydrophobic extra cellular layer made up of two main components, cutin and cuticular waxes. Cutin is a polymeric web made up primarily of C_{16} and C_{18} hydroxy fatty acids linked by ester bonds.[1] Depolymerization allows for analysis of cutin monomers and has revealed differences in cutin composition and amount between species and during plant development.[2, 3]

Cuticular wax composition varies both between species and between organs within one plant. Most plants' waxes consist largely of very long chain fatty acids (VLCFAs) and their derivatives including alcohols, aldehydes, alkanes, and ketones occurring as homologous series. Chain lengths can range from C_{20} to C_{40}, but not all chain lengths (or all compound classes) are represented in all plants. A small range of chain lengths usually predominates in all compound classes but this too varies between species. In addition to the compounds already mentioned, many species contain esters of C_{16} to C_{34} fatty acids with the C_{20} to C_{36} primary alcohols, yielding chain lengths ranging from C_{36} to C_{70}. Some plants also contain cyclic compounds in their cuticular wax. For example, rye leaves contain alkylresorcinols[4] while the cuticular waxes of *Kalanchoe* species, tomatoes, cherries, peppers, and *Macaranga* species contain various pentacyclic triterpenoids.[5-9] Wax composition does not always remain constant throughout development of a plant or organ, and changes in absolute and relative wax coverage can be substantial.[10, 11]

Cuticular waxes are both embedded in the cutin and external to the cutin/wax layer. Waxes embedded in the cutin are referred to as intracuticular waxes. Overlying the intracuticular wax layer is the epicuticular wax which can be either a smooth film or a film with protruding wax crystals (fig.1.1). This epicuticular wax layer can be removed by cryo-adhesion using water or adhesion with gum arabic[12] enabling separate chemical analyses of epicuticular and intracuticular waxes. The intracuticular wax can be removed by treatment with chloroform or hexane after the epicuticular wax has been peeled off.

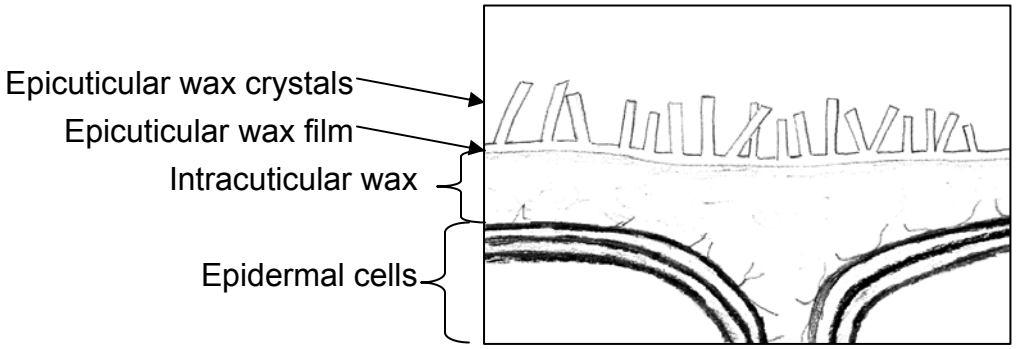

Figure 1.1: Cross section showing structure of the plant cuticle: Epicuticular wax crystals are not present in all species. Diagram is not to scale

1.2: Epicuticular vs. Intracuticular wax composition

Comparisons of the epicuticular and intracuticular waxes have revealed that in *Taxus baccata*, longer chain VLCFA compounds are found preferentially in the epicuticular wax layer while shorter chains are found in the intracuticular wax. A difference was also found in the distribution of compound classes, with alcohols, fatty acids, aldehydes, and alkyl esters predominating in the epicuticular wax layer while tocopherols and aromatic esters were localized to the intracuticular wax.[13] In species containing triterpenoids as well as VLCFA derivatives, the VLCFA derivatives tend to be found primarily in the epicuticular wax, and triterpenoids in the intracuticular wax.[12, 14] Most attempts to explain the distribution of compounds among the wax layers have relied on differences in polarity or geometry between the polycyclic triterpenoids and the linear VLCFA derivatives perhaps affecting their relative mobility within the cuticle. However, in some cases triterpenoids obviously get all the way to the epicuticular wax and are present in epicuticular wax crystals.[9]

In some species with epicuticular wax crystals, the chemical composition of the crystals has been confirmed. The use of scanning electron microscopy (SEM) combined with chemical analysis has enabled some links to be established between the chemical composition of the crystals and their physical form. SEM examination of numerous species has demonstrated the fact that crystals come in a variety of forms including threads, plates, and rodlets.[15, 16] Comprehensive chemical analyses of epicuticular wax crystals have been less exhaustive, but provide essential information.

Characteristic crystals tend to form when there is a high concentration of a single component within the epicuticular wax. It appears likely that there is some threshold level above which crystals can (or must) form, but it is unclear whether this threshold is in absolute amount of a compound (in $\mu g/cm^2$) or its relative concentration with respect to other wax components. Distinctive tubular crystals are associated with high concentrations of nonacosan-10-ol[13] while transversely ridged rodlets can be composed of either ketones or alkanes.[17] Triterpenoid containing crystals of various forms have been reported. Threads on the stems of *Macaranga* species contain high concentrations of epi-taraxerol and taraxerone[9] while plates on *Vitis vinifera* contain oleanolic acid and *Malus prunus* displays plates and greasy films of ursolic acid.[16] Other triterpenoids have been found in the form of platelets and polygonal rodlets.[9, 15, 16] Experiments have, in some cases, confirmed the ability of a single compound or combination of compounds to form crystals in vitro that resemble those seen on the plant.[17, 18] In other cases, however, attempts to recrystallize extracted waxes or wax components have been unsuccessful. Thus linking chemical composition to physical appearance is based on observation of the native plant surface with SEM and chemical analysis of the physically removed epicuticular wax crystals. It should be noted that in some cases epicuticular wax removal includes the epicuticular wax film as well as the crystals, thus complicating the analysis.

1.3: Cuticle Function

The primary function of the cuticle is generally accepted to be one of protecting against non-stomatal water loss.[19] Some studies have shown that decreased wax loads can lead to increased permeability[20] but there is no evidence that increased wax load

leads to a better barrier against water loss.[21] The composition of the cuticle has also been implicated in its effectiveness as a transpiration barrier.[22]

The cuticle has been shown to have self cleaning properties by virtue of its non-wettability in addition to its microstructure.[23] This "lotus effect" has been demonstrated in several plants[24] and effectively describes the cuticle's ability to enhance the cleaning role of water.

Another key role of the cuticle is in protecting the plant from ultraviolet radiation. Research with isolated cuticles from leaves and fruits of woody species has shown that the cuticle strongly attenuates light in the UV-B region of the spectrum (280 – 320 nm) while transmitting almost all of the visible light.[25] More recent experiments with isolated cuticular waxes of the dwarf mountain pine have found that even when reconstituted after extraction, waxes absorb strongly in the UV region.[26]

The plant surface, covered in epicuticular wax, mediates interactions between the plant and the outside world. Research has shown that cuticle composition in rose leaves is correlated with susceptibility to fungal infection.[27] Cuticular wax components also help to protect the plant from insects, having been implicated as deterrents to insect feeding[28] as well as affecting movement, oviposition, and other behaviours. [29]

In some species of *Macaranga*, epicuticular waxes have been shown to play a role in selectively preventing most ant species from accessing the plant while allowing partner ants (which have a symbiotic relationship with the host plant) to climb up the stem.[9] Epicuticular waxes are also crucial in the "slippery zone" of pitchers in some carnivorous plants and ensure that insects cannot climb out of the trap once they have been lured into the plant.[30]

1.4: Role of cuticular components

Studies on tomato (*Lycopersicon esculentum*) have attempted to locate the transpiration barrier to a specific part of the cuticle. Vogg and colleagues found that transpiration was not affected by removal of the epicuticular wax layer from wild type fruits, but a mutant cuticle deficient in intracuticular alkanes and aldehydes over 30 carbons in length had a much higher permeability than the wild type cuticle (after removal of epicuticular wax).[22] This gives some clues about the role of VLCFA

derivatives in the intracuticular wax, implying that these compounds contribute to the vapour barrier properties of the intracuticular wax layer. These results also suggest that the epicuticular wax does not play a role in preventing water loss.

Although insights can be gained by the study of mutants with wax specific deficiencies, these studies cannot answer all of the questions raised by the diversity and complexity of cuticle compositions found in nature. Many questions remain unanswered regarding the function of alkanes and other aliphatic compounds in the epicuticular wax, the role of triterpenoids in the cuticle, and how compounds end up in chemically distinct epicuticular and intracuticular wax layers.

1.5: Synthesis and elongation of fatty acids

VLCFAs and their derivatives are important components of cuticular wax. Their synthesis begins with the process of fatty acid synthesis (FAS), a process that occurs in all cells. De novo synthesis of fatty acids (up to C_{18}) takes place through a repeated cycle of four steps (fig.1.2). The four steps are: Condensation of a C_2 unit from malonyl-ACP onto an existing acyl – ACP molecule, reduction of the β-ketoacyl – ACP to β -hydroxyacyl ACP, dehydration of the β -hydroxyacyl – ACP to *trans*-Δ^2-enoyl – ACP, and finally a second reduction step in which the *trans*-Δ^2-enoyl – ACP is reduced to an acyl-ACP. From here, the initial chain (now two carbons longer) is joined to another malonyl-ACP and the cycle is repeated, eventually producing a saturated C_{16} or C_{18} chain.[31]

For further elongation, the long chain fatty acids are esterified to Coenzyme A (CoA). The fatty acid thioesters can then be used in glycerolipid synthesis, cutin biosynthesis, or used for further elongation (to VLCFAs). The elongation of long chain fatty acids to VLCFAs is analogous to the process of fatty acid synthesis. However, in the case of fatty acid elongation, the two-carbon donor unit is malonyl-CoA rather than malonyl-ACP as in the case of FAS. Fatty acid elongation is performed by several fatty acid elongation enzyme complexes (FAE). The four steps of condensation, reduction, dehydration, and further reduction extends the chain by two carbons per cycle. [32] Chain lengths from C_{20} to C_{40} are produced by fatty acid elongation, and it has been shown that the condensing enzyme in each FAE shows some chain length specificity. The other

three enzymes involved appear to be non-selective and can accept substrates of a variety of chain lengths. [33]

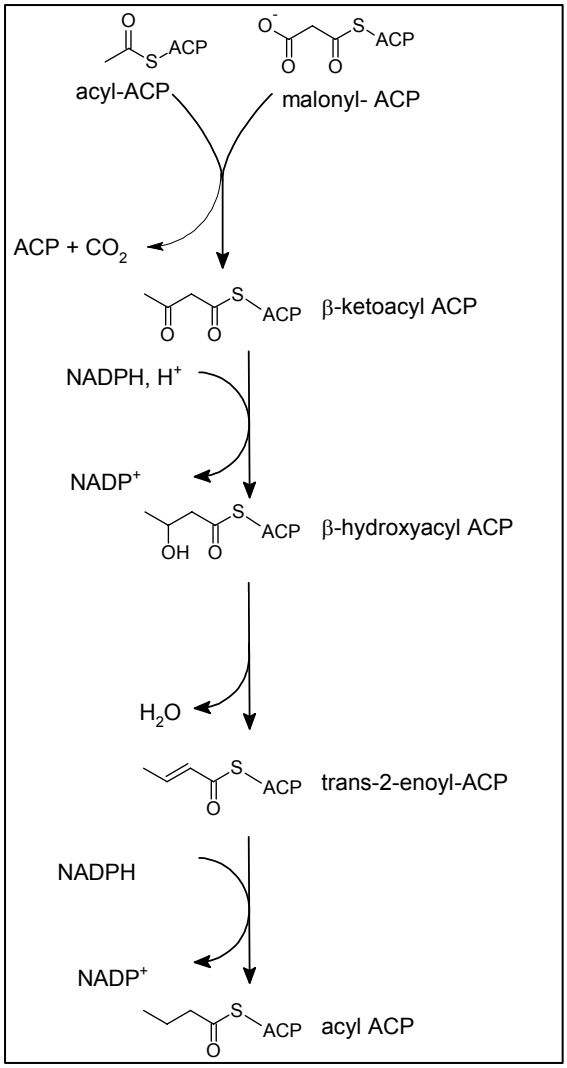

Figure 1.2: Fatty acid synthesis (FAS) pathway: The four steps of elongation, reduction, dehydration, and further reduction add two carbons. The cycle is repeated, until a chain length of C_{16} or C_{18} is obtained. The entire reaction series takes place within the plastid.

1.6: Modification of very long chain fatty acids

VLCFAs are modified to produce the very long chain components of cuticular waxes. One pathway involved in this process is the acyl-reduction pathway whereby fatty acids are reduced to form primary alcohols with the same chain length as the fatty acid precursor. Primary alcohols produced by this pathway can then either be combined with VLCFAs to produce alkyl esters or may exist as free alcohols. The other pathway

that has been proposed is the decarbonylation pathway. In this pathway, fatty acids are reduced to form aldehydes which are then decarbonylated to form alkanes with a characteristic chain length one carbon shorter than the aldehyde precursor. Oxidation of alkanes along this pathway can form secondary alcohols which can then be further oxidized to form ketones. [32]

As a result of elongation and modification pathways, VLCFA derivatives have characteristic chain length distributions which differ by compound class. The fatty acids, primary alcohols and aldehydes are dominated by homologs with even chain lengths while the alkanes, secondary alcohols, and ketones are dominated by odd chain lengths. The presence of these dominant chain lengths can be explained by the successive two-carbon additions during elongation and the loss of a single carbon in the decarbonylation pathway. Less obvious is the reason for the presence of the minor chain lengths, which are sometimes present in appreciable amounts. The normal extender unit in both FAS and fatty acid elongation is a malonyl unit which adds two carbons to the chain in each cycle, so achieving an irregular chain length by following this "normal" route would be impossible. It would only be by starting with an uneven chain length (for example propionyl-ACP) at the beginning of FAS and adding two carbons per cycle that an irregular chain length could be obtained. Achieving an irregular chain length by the use of an "abnormal" extender unit would be improbable, because loss of CO_2 in the initial condensation step requires the presence of a β-keto group in the extender unit, which is only possible if the extender unit has an odd chain length.

Researchers have been able to identify several of the genes involved in VLCFA modification using molecular genetics approaches.[32] While this has led to the identification and characterization of some genes and enzymes involved in wax biosynthesis, many more are still unknown. The fact that this method relies on investigation of wax mutants (frequently from Arabidopsis) which are often visually screened could be a limitation. Effects such as glossy vs. glaucous stems, organ fusions, or dwarf plants are easy to identify, but changes in wax chemistry may not always produce a visually discernable phenotype. Other problems could be caused by redundancy, in which another gene (either an allele or a related gene) could make up for any deficit caused by disabling the gene of interest, thus masking the effect.

No less significant than their synthesis is the transport of fatty acids and their derivatives to the plant surface. For large hydrophobic molecules to pass through the cytoplasm, cell wall, and cell membrane obviously requires assisted transport. Some of the genes and proteins involved in this export have been identified and their roles partially characterized, [34, 35] but the process is not fully understood.

1.7: Cuticular triterpenoid biosynthesis

The biosynthesis of terpenoids proceeds according to the biogenetic isoprene rule first proposed in 1959 by Ruzicka and colleagues. According to the biogenetic isoprene rule, the preferred structure of any cyclic terpene could be explained mechanistically by the cyclization of a precursor such as geraniol, farnesol, geranylgeraniol , or squalene. [36]

Each branched chain terpenoid precursor is a product of successive additions of five-carbon units (fig. 1.3). The first two five-carbon units are dimethylallyl diphospate (DMAP) and its isomer isopentenyl diphosphate (IPP). These can be produced from acetyl CoA precursors via the mevalonate pathway or from D-glyceraldehyde-3-phosphate and pyruvate via the DOXP/MEP pathway.[37] Condensation of DMAP and IPP yields geranyl diphosphate (GPP), which is converted to farnesyl diphosphate (FPP) by the addition of another IPP unit. Head to head condensation of two FPP molecules yields squalene which is converted to 2,3-oxidosqualene by squalene epoxidase.

Triterpenoids are formed by the cyclization of 2,3-oxidosqualene under the tight control of one of a family of oxidosqualene cyclases (OSCs). Each triterpenoid synthase enzyme may be responsible for the production of one or several triterpenoid products. Although the process is described here in two parts (cyclization followed by methyl and hydrogen shifts), the entire process, from 2,3-oxidosqualene to the triterpenoid product, is under the control of the same enzyme.

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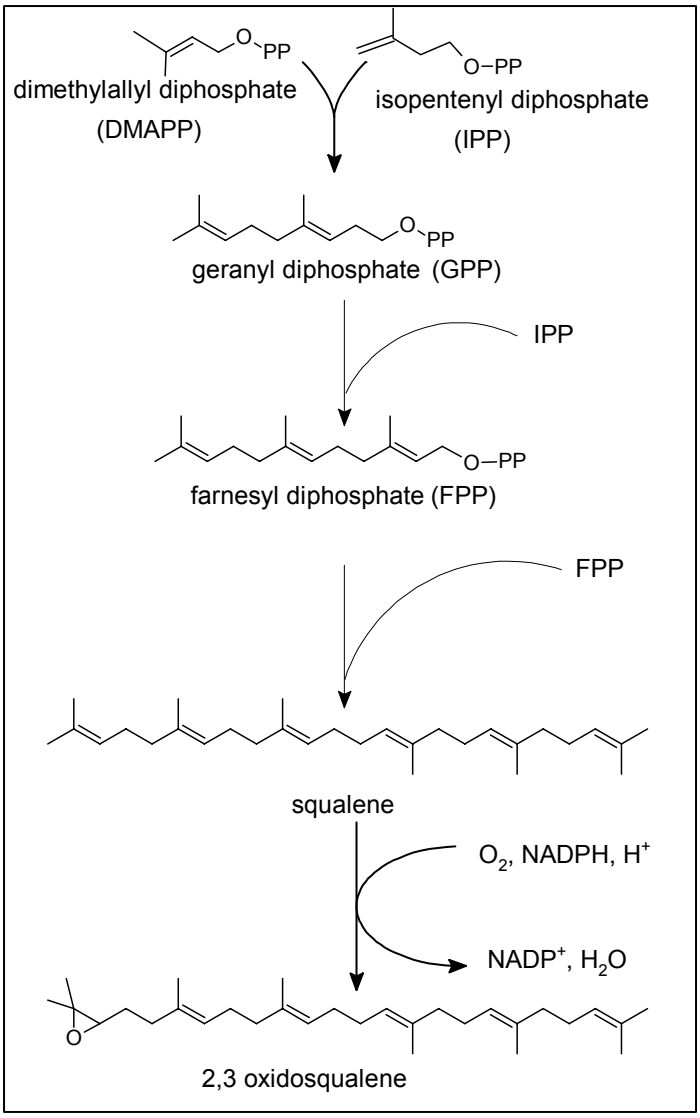

Figure 1.3: Biosynthetic pathway leading to 2,3-oxidosqualene: Successive additions of five-carbon units yield farnesyl diphosphate. Head to head condensation of two FPP units yields squalene which is converted to 2,3-oxidosqualene. Enzymes are not shown, but each step is catalyzed by a unique enzyme. 2,3 oxidosqualene is the last common precursor leading to sterols (primary metabolism) and triterpenoids (secondary metabolism) in plants.

In the biosynthesis of triterpenoids, 2,3-oxidosqualene is cyclized in a chair, chair, chair conformation, whereas the synthesis of steroids requires a chair, boat, chair arrangement. The cascade of cyclizations is initiated by protonation of the epoxide by an acidic residue, forming a carbocation which is attacked by a π-electron system, closing the first ring and forming another carbocation (fig. 1.4).

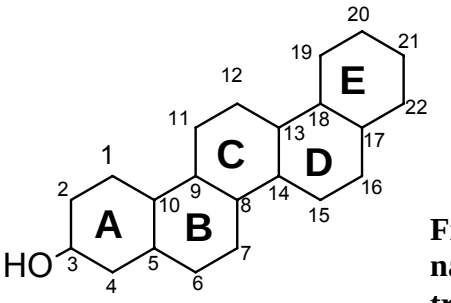

Figure 1.4: Standard numbering and ring naming convention for pentacyclic triterpenoid core

This series of steps continues, forming the first three rings and a five membered D ring in the dammarenyl cation. (fig. 1.5) From here, the cation can be deprotonated, leading to the tetracyclic dammarane type tritpernoids, or the fourth ring can be either expanded and a fifth ring closed, leading to the lupenyl cation. From the lupenyl carbocation, deprotonation leads to lupeol, with a five membered E ring, while ring expansion by attack of the π-electron system forms the germanicyl carbocation. From here, deprotonation forms germanicol, or the carbocation can undergo anywhere from one to eight methyl and/or hydrogen shifts before deprotonation occurs to yield a triterpenoid product (fig. 1.6).[38]

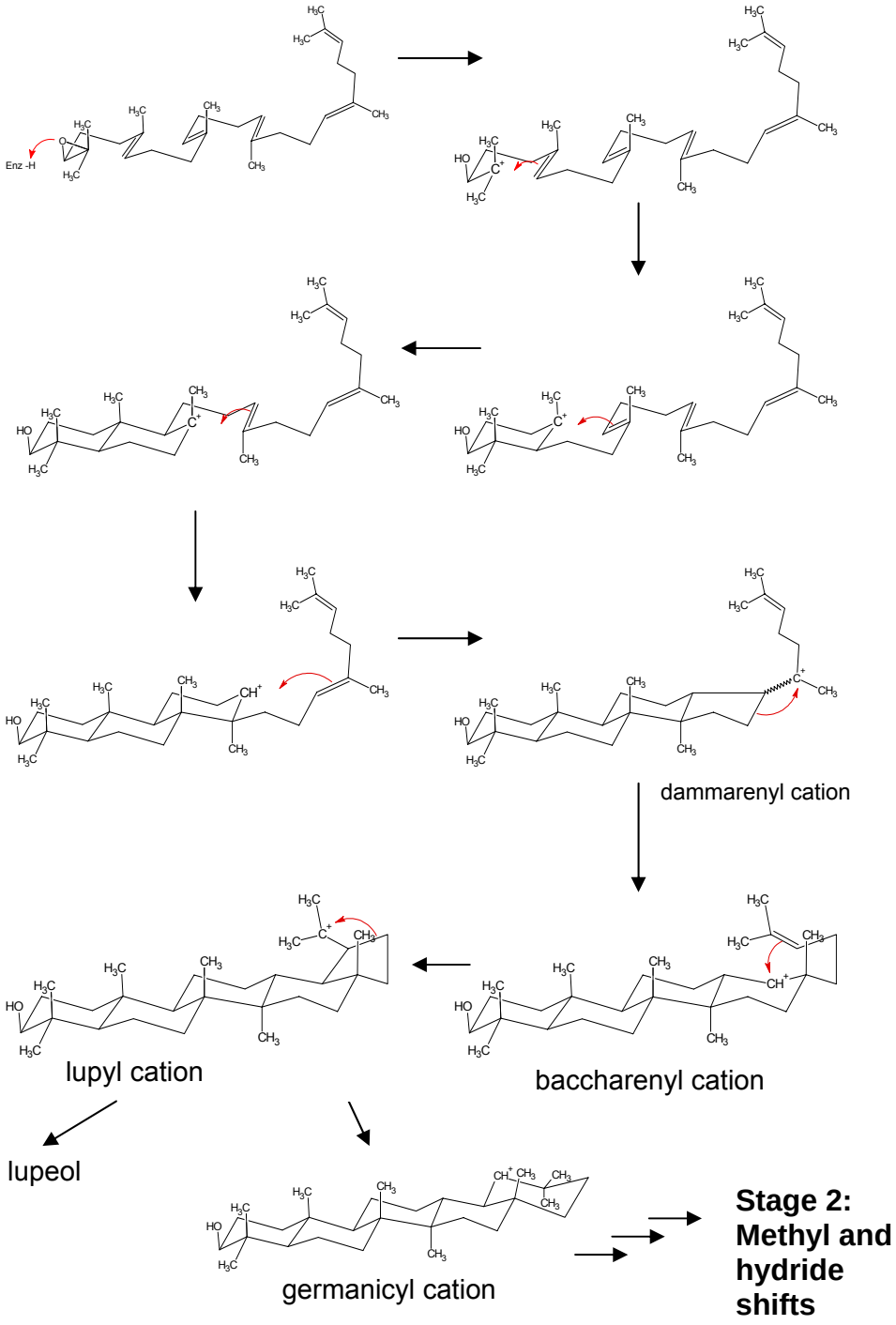

Figure 1.5: Stage 1 of the cyclization of 2,3 oxidosqualene to form pentacyclic triterpene skeleton: After activation at the epoxide, a series of π-electron attacks close the A-D rings. Formation and expansion of the E ring is also shown.

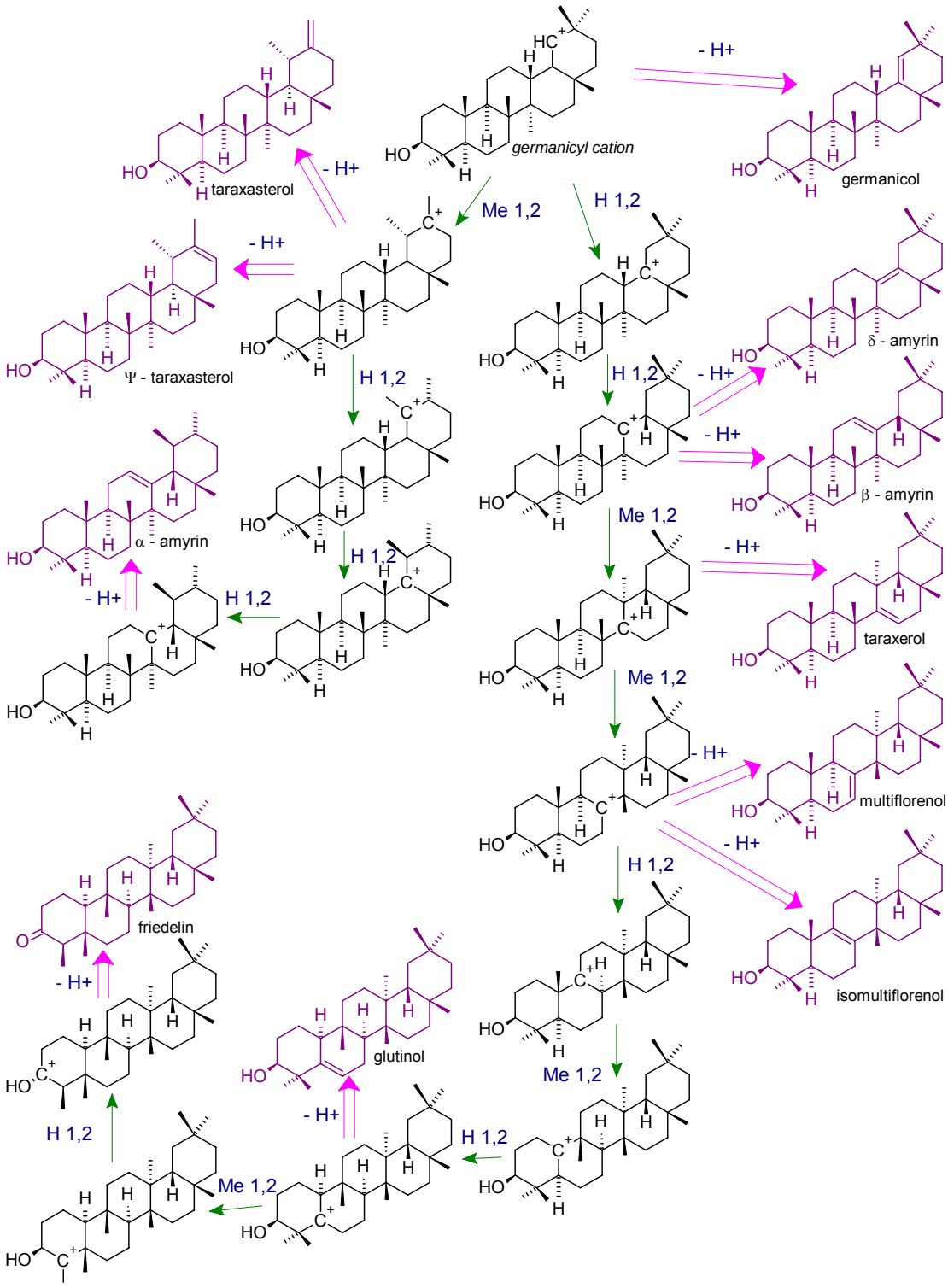

Figure 1.6: Stage 2 of cyclization forming pentacyclic triterpenoids: Hydride and methyl shifts are shown by green arrows, deprotonations leading to triterpenoid products (in purple) are shown by pink arrows. Proposed mechanism modelled after description in Xu (2004)[38] .

1.8: Triterpenoid synthases

To produce friedelin or glutinol, which may be classified as the most "complex" of pentacyclic triterpenoids, the positive charge migrates from ring E to ring A in a series of 1,2 methyl and hydrogen shifts.[38] The entire cyclization process to produce the pentacyclic structure, along with the methyl and hydride shifts leading to eventual deprotonation are thought to be under the control of a single triterpenoid synthase enzyme. This is reasonable when considered in light of the many triterpenoid synthase genes which have been characterized and found to code for enzymes that produce a wide variety of triterpenoids from 2,3-oxidosqualene.[39]

Based on the crystal structure of human oxidosqualene cyclase[40] which converts oxidosqualene into lanosterol, predictions can be made about the mechanism and active site of triterpenoid synthases in plants. Based on the enzymes that have been fully characterized, the active site appears to be lined with aromatic residues, whose high π-electron density stabilizes the positive charge while it is migrating through the forming triterpenoid.[41]

If this is true, friedelin synthase would need to stabilize extensive charge migration as the positive charge migrates back from C_{19} to C_3 in numerous shifts before deprotonation. This is after stabilizing the positive charge during the first phase of triterpenoid formation (fig. 1.5) in which 2,3-oxidosqualene is protonated at the epoxide and cyclizes by a series of π-electron attacks, moving the positive charge from C_2 of oxidosqualene (C_4 in triterpenoid numbering) to C_{19}. While the first phase (cyclization) is common to all pentacyclic triterpenoid biosynthesis, the degree of charge migration through 1,2 methyl and hydride shifts varies depending on the final product. A comparison of the friedelin synthase structure with those of other oxidosqualene cyclases may help elucidate not only the biosynthesis of friedelin, but the biosynthesis of triterpenoids in general.

Oxidosqualene cyclases from a number of plant species have been cloned and characterized by heterologous expression in yeast. Among the triterpenoid synthases that have been identified and characterized in this way are several lupeol synthases, numerous β-amyrin synthases, and a number of mixed function triterpenoid synthases yielding a

variety of products including germanicol, taraxasterol, α-amyrin, and ψ-taraxasterol among others.[39] However, up to this point neither a friedelin nor a glutinol synthase has been characterized. Cloning and characterization of either or both of these genes would yield interesting information about the cyclization mechanism of triterpenoid synthases due to the extensive charge stabilization necessary in their biosynthesis. (fig. 1.6)

1.9: Cuticular triterpenoid diversity

While all triterpenoids share a common precursor (2,3-oxidosqualene) and chemical formula ($C_{30}H_{50}O$), their structures vary widely (fig. 1.6), as does their distribution. Some triterpenoids are widespread, such as α and β-amyrin, which are found in the cuticular wax of azalea and oak leaves as well as the cuticles of tomato, bell pepper and eggplant fruits, as well as other plants.[8, 42-44] Other triterpenoids are not as widely distributed. Friedelin, for example, has been identified in the cuticular wax of some *Kalanchoe* species[5] as well as bell peppers,[8] but is not nearly as ubiquitous as the α and β-amyrins. Novel triterpenoids not found in plant extracts have been identified and characterized by expressing candidate plant genes in yeast,[45, 46] opening up new avenues of exploration for triterpenoid chemistry.

1.10: *Kalanchoe daigremontiana*

Kalanchoe daigremontiana (fig. 1.7) is a member of the Crassulaceae family native to Madagascar. A succulent plant with an upright growth habit, *K. daigremontiana* reproduces mainly vegetatively, with plantlets growing in the notches of the leaves on the parent plant.[47] Plantlets can be easily detached and grown in soil, giving rise to the plant's common name of "mother of thousands". It has been observed that plantlets can grow on the margins of other plantlets that are still attached to a parent plant, giving rise to three generations on the same plant. Plantlets can arise not only on the margins of leaves still attached to the plant but even on leaves which have been detached from the plant and thus have no supply of water or nutrients.[47]

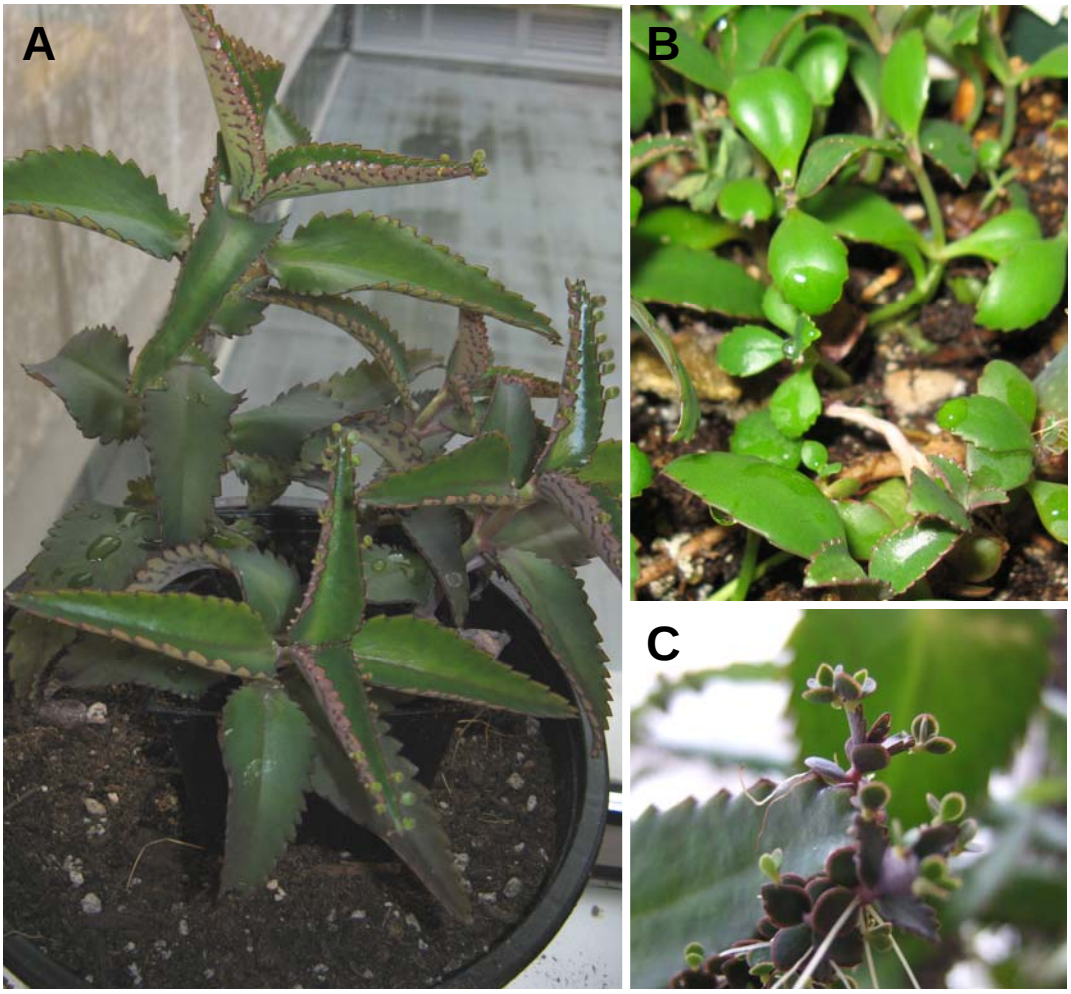

Figure 1.7: *Kalanchoe daigremontiana*: A) three *K. daigremontiana* plants; B) several plantlets; C) plantlets growing on the leaf margins of other plantlets

K. daigremontiana grows with a decussate plant architecture in which leaves are paired and grow symmetrically, with each pair rotated 90 degrees with respect to the pair below. The first two leaf pairs of the plant are different to the subsequent leaves. These first four leaves appear while the plantlet is attached to the parent plant and have continuous leaf margins. These leaves never get very large, reaching a maximum length of less than 1cm. In contrast, the third and higher leaf pairs have serrated leaf margins, grow larger, and are capable of producing plantlets.[47]

1.11*: K. daigremontiana* wax composition

A comprehensive analysis of the waxes of *K. daigremontiana* could not be found in the literature, but several other *Kalanchoe* species have been studied and provide some

background information. Pentacyclic triterpenoids were reported in the genus *Kalanchoe* for the first time in 1972 when researchers in England found α and β-amyrins in the leaf material of *K. pinnata*.[48] This analysis was done on pulverized leaf material extracted with petroleum, so the triterpenoids could not be localized to the cuticle. Further analysis on the same species in 1974[49] identified VLCFA components including C_{31} and C_{33} alkanes, C_{16} to C_{26} fatty acids, and C_{26} to C_{36} alcohols. Rarer triterpenoids, including friedelin, glutinol, and taraxerol, were identified in the flowers of *K. spathulata* a short time later.[50] Work in the 1990s on several *Kalanchoe* species (*K. fedtschenkoi, K. gastonis-bonnieri, K. marnieriana, K. miniata, K. pumila, K. thyrsiflora*) identified triterpenoids including friedelin, glutin, glutinone, and β-amyrenone in their cuticular waxes.[5] Thus various species within the genus are known to contain triterpenoids, some conclusively within the cuticular wax, and others possibly so.

Taken together, these prior analyses of *Kalanchoe* species indicated a strong possibility of finding triterpenoids in the wax of *K. daigremontiana*, which was readily available. Preliminary chemical analysis of *K. daigremontiana* (Jetter lab, unpublished results) had found relatively high concentrations of triterpenoids, particularly glutinol and friedelin, which are both considered "late" triterpenoids in terms of the proposed mechanism of triterpenoid formation (fig. 1.6). The relatively high wax content, high relative triterpenoid concentration, and ease of propagation of *K. daigremontiana* made it an obvious target for further investigation.

1.12: Scope of thesis work

The ultimate goal of identifying and characterizing a friedelin and/or glutinol synthase led to the identification of *K. daigremontiana* as a model system for further investigation. In light of the ultimate goal of the larger project, more immediate objectives were established. As a first step towards characterization of a triterpenoid synthase from *K. daigremontiana*, an understanding of its wax composition was required. Preliminary chemical analysis suggested differences between the cuticular wax composition of plantlets vs. mature leaves, but a systematic investigation of how the wax composition changed over the course of leaf development had not been carried out. The localization of triterpenoids within the cuticle was also of interest to assist in

understanding how compounds end up in chemically distinct epicuticular and intracuticular layers as well as how crystal form and chemistry are related.

The project had several objectives, all of which tied into the larger goal of better understanding the chemistry of plant cuticular waxes. Work was focussed on *K. daigremontiana* in two distinct investigations:

a) An investigation of cuticular wax composition over the course of leaf development
b) An examination of the chemical composition of the epicuticular and intracuticular wax on *K. daigremontiana* as well as an examination of epicuticular crystals under SEM

Within each experiment, there was an eventual goal of tying the findings back to what was already known about wax biosynthesis as well as the work on triterpenoid synthase characterization.

Attempts were also made to characterize several putative triterpenoid synthases by heterologous expression in yeast. These experiments met with little success and are reported in appendix 1 (pg. 76). It is hoped that some of the findings of the chemical analysis will allow more insight when approaching the biological and biochemical avenues of this project. Work on gene characterization continues in the lab.

References

(1) Kolattukudy, P. E. *Science* **1980**, *208*, 990-1000.

(2) Riederer, M.; Schonherr, J. *Planta* **1988**, *174*, 127-138.

(3) Heredia, A. *Biochim. Biophys. Acta, Gen. Subj.* **2003**, *1620*, 1-7.

(4) Ji, X. F.; Jetter, R. *Phytochemistry* **2008**, *69*, 1197-1207.

(5) Siems, K.; Jas, G.; Arriagaginer, E. J.; Wollenweber, E.; Dorr, M. *Z. Naturforsch., C: J. Biosci.* **1995**, *50*, 451-454.

(6) Bauer, S.; Schulte, E.; Thier, H. P. *Eur. Food Res. Tech.* **2004**, *219*, 223-228.

(7) Peschel, S.; Franke, R.; Schreiber, L.; Knoche, M. *Phytochemistry* **2007**, *68*, 1017-1025.

(8) Bauer, S.; Schulte, E.; Thier, H. P. *Eur. Food Res. Tech.* **2005**, *220*, 5-10.

(9) Markstadter, C.; Federle, W.; Jetter, R.; Riederer, M.; Holldobler, B. *Chemoecology* **2000**, *10*, 33-40.

(10) Bringe, K.; Schumacher, C. F. A.; Schmitz-Eiberger, M.; Steiner, U.; Oerke, E. C. *Phytochemistry* **2006**, *67*, 161-170.

(11) Jetter, R.; Schaffer, S. *Plant Physiol.* **2001**, *126*, 1725-1737.

(12) Jetter, R.; Schaffer, S.; Riederer, M. *Plant Cell Env.* **2000**, *23*, 619-628.

(13) Wen, M.; Buschhaus, C.; Jetter, R. *Phytochemistry* **2006**, *67*, 1808-1817.

(14) Buschhaus, C.; Herz, H.; Jetter, R. *New Phytol.* **2007**, *176*, 311-316.

(15) Barthlott, W.; Neinhuis, C.; Cutler, D.; Ditsch, F.; Meusel, I.; Theisen, I.; Wilhelmi, H. *Bot. J. Linn. Soc.* **1998**, *126*, 237-260.

(16) Jeffree, C. E. In *Fine structure of the plant cuticle;* Riederer, M., Muller, C., Eds.; Biology of the Plant Cuticle; Blackwell Publishing: UK, 2006; Vol. 23, pp 11-110.

(17) Meusel, I.; Neinhuis, C.; Markstadter, C.; Barthlott, W. *Can. J. Botany* **1999**, *77*, 706-720.

(18) Jetter, R.; Riederer, M. *Planta* **1994**, *195*, 257-270.

(19) Jeffree, C. E. In *The cuticle, epicuticular waxes and trichomes of plants, with reference to theirstructure, functions and evolution;* Juniper, B., Southwood, R., Eds.; Insects and the Plant Surface; E. Arnold: London, 1986; pp 23-135.

(20) Jenks, M. A.; Joly, R. J.; Peters, P. J.; Rich, P. J.; Axtell, J. D.; Ashworth, E. N. *Plant Physiol.* **1994**, *105*, 1239-1245.

(21) Riederer, M.; Schreiber, L. *J. Exp. Bot.* **2001**, *52*, 2023-2032.

(22) Vogg, G.; Fischer, S.; Leide, J.; Emmanuel, E.; Jetter, R.; Levy, A. A.; Riederer, M. *J. Exp. Bot.* **2004**, *55*, 1401-1410.

(23) Barthlott, W.; Neinhuis, C. *Planta* **1997**, *202*, 1-8.

(24) Neinhuis, C.; Barthlott, W. *Ann. Botany* **1997**, *79*, 667-677.

(25) Krauss, P.; Markstadter, C.; Riederer, M. *Plant Cell Env.* **1997**, *20*, 1079-1085.

(26) Jacobs, J. F.; Koper, G. J. M.; Ursem, W. N. J. *Prog.Org.Coat.* **2007**, *58*, 166-171.

(27) Goodwin, S. M.; Edwards, C. J.; Jenks, M. A.; Wood, K. V. *Hort. Science* **2007**, *42*, 1631-1635.

(28) Eigenbrode, S. D.; Pillai, S. K. *J. Chem. Ecol.* **1998**, *24*, 1611-1627.

(29) Eigenbrode, S. D.; Espelie, K. E. *Annu. Rev. Entomol.* **1995**, *40*, 171-194.

(30) Gorb, E.; Haas, K.; Henrich, A.; Enders, S.; Barbakadze, N.; Gorb, S. *J. Exp. Biol.* **2005**, *208*, 4651-4662.

(31) Kunst, L.; Jetter, R.; Samuels, A. L. In *Biosynthesis and transport of plant cuticular waxes;* Riederer, M., Muller, C., Eds.; Biology of the Plant Cuticle; Blackwell Publishing: UK, 2006; Vol. 23, pp 182-207.

(32) Kunst, L.; Samuels, A. L. *Prog. Lipid Res.* **2003**, *42*, 51-80.

(33) Millar, A. A.; Kunst, L. *Plant J.* **1997**, *12*, 121-131.

(34) Pighin, J. A.; Zheng, H. Q.; Balakshin, L. J.; Goodman, I. P.; Western, T. L.; Jetter, R.; Kunst, L.; Samuels, A. L. *Science* **2004**, *306*, 702-704.

(35) Bird, D.; Beisson, F.; Brigham, A.; Shin, J.; Greer, S.; Jetter, R.; Kunst, L.; Wu, X. W.; Yephremov, A.; Samuels, L. *Plant J.* **2007**, *52*, 485-498.

(36) Eschenmoser, A.; Arigoni, D. *Helv. Chim. Acta* **2005**, *88*, 3011-3050.

(37) Lichtenthaler, H. K. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1999**, *50*, 47-65.

(38) Xu, R.; Fazio, G. C.; Matsuda, S. P. T. *Phytochemistry* **2004**, *65*, 261-291.

(39) Phillips, D. R.; Rasbery, J. M.; Bartel, B.; Matsuda, S. P. T. *Curr. Opin. Plant Biol.* **2006**, *9*, 305-314.

(40) Thoma, R.; Schulz-Gasch, T.; D'Arcy, B.; Benz, J.; Aebi, J.; Dehmlow, H.; Hennig, M.; Stihle, M.; Ruf, A. *Nature* **2004**, *432*, 118-122.

(41) Morikubo, N.; Fukuda, Y.; Ohtake, K.; Shinya, N.; Kiga, D.; Sakamoto, K.; Asanuma, M.; Hirota, H.; Yokoyama, S.; Hoshino, T. *J. Am. Chem. Soc.* **2006**, *128*, 13184-13194.

(42) Wang, Y. F.; Braman, S. K.; Robacker, C. D.; Latimer, J. G.; Espelie, K. E. *J. Am. Soc. Hort. Sci.* **1999**, *124*, 239-244.

(43) Martins, C. M. C.; Mesquita, S. M. M.; Vaz, W. L. C. *Phytochem. Anal.* **1999**, *10*, 1-5.

(44) Leide, J.; Hildebrandt, U.; Reussing, K.; Riederer, M.; Vogg, G. *Plant Physiol.* **2007**, *144*, 1667-1679.

(45) Fazio, G. C.; Xu, R.; Matsuda, S. P. T. *J. Am. Chem. Soc.* **2004**, *126*, 5678-5679.

(46) Lodeiro, S.; Xiong, Q. B.; Wilson, W. K.; Kolesnikova, M. D.; Onak, C. S.; Matsuda, S. P. T. *J. Am. Chem. Soc.* **2007**, *129*, 11213-11222.

(47) Johnson, M. A. *Bull. Tor. Bot. Club* **1934**, *61*, 355-366.

(48) Gaind, K. N.; Gupta, R. L. *Phytochemistry* **1972**, *11*, 1500-&.

(49) Gaind, K. N.; Gupta, R. L. *Planta Med.* **1974,** *25*, 193-197.

(50) Gaind, K. N.; Singla, A. K.; Boar, R. B.; Copsey, D. B. *Phytochemistry* **1976,** *15*, 1999-2000.

(51) Gniwotta, F.; Vogg, G.; Gartmann, V.; Carver, T. L. W.; Riederer, M.; Jetter, R. *Plant Physiol.* **2005,** *139*, 519-530.

Chapter 2 – Cuticular wax composition in *Kalanchoe daigremontiana* during leaf ontogenesis

A version of this chapter has been submitted for publication. van Maarseveen, C.; Han, H.; and Jetter, R. Development of the cuticular wax during growth of *Kalanchoe daigremontiana* (Hamet et Perr. de la Bathie) leaves

2.1: Introduction

In the quest to characterize a friedelin or glutinol synthase, *K. daigremontiana* had been identified as a plant of interest based on the presence of both friedelin and glutinol in the cuticular wax of other *Kalanchoe* species.[1, 2] Preliminary analysis of cuticular wax from *K. daigremontiana* gave promising results. The availability and ease of propagation of the species were added advantages.

To fully characterize the enzyme and gene responsible for friedelin synthesis, it is necessary to determine when and where the gene is being expressed. This can be accomplished indirectly through analysis of triterpenoid accumulation patterns in the cuticular wax. A knowledge of when and where friedelin/glutinol appears in the cuticle and how its concentration changes during leaf ontogenesis can assist in selecting tissues in which the gene is being expressed. Active transcription gives rise to mRNA which can be used in RT-PCR to access cDNA sequences of interest. After these genes have been cloned, expressed, and characterized, the enzymes associated can be localized and their activity measured. Eventually this can be correlated with chemical accumulation data, allowing a better understanding of cuticular triterpenoid biosynthesis and export.

Time course analyses of the cuticular waxes of several species have revealed changes in wax composition over time. The cuticle of the sweet cherry fruit, which, like many other fruits, displays a high concentration of triterpenoids, shows a decrease in the relative concentration of triterpenoids and increase in the relative concentration of alkanes as the fruit matures.[3] A similar pattern is seen in 'MicroTom' tomatoes.[4] A similar time course study of cuticular waxes in the leaves of *K. daigremontiana* had not been carried out, therefore, the goals of the current project were:

a) To establish a reliable growth curve for different leaves of *K. daigremontiana*

b) To extract and analyze the cuticular wax at different time points during leaf development to determine how wax composition changed during development and, in particular, when triterpenoids accumulated.

2.2: Materials and methods

Plant cultivation and growth is described as well as procedures for isolation and chemical analysis of cuticular wax.

2.2.1: Plant material

K. daigremontiana plants were maintained in the University of British Columbia greenhouse. Several small plants were re-potted from the greenhouse and grown on the laboratory windowsill in ambient conditions. Plantlets in the same stage of development were harvested from the margins of approximately seven leaves, planted in soil, and watered approximately every 3-4 d. Leaf length measurements (base to tip) were made on these plants during the spring and summer of 2007 and all measurements were corrected for leaf age rather than plant age to facilitate comparison between different leaf pairs. To this end, leaf age was counted from a day 0 defined by the first visible appearance of the new leaflets (typically corresponding to a length of less than 1 mm). The average plant age was <7 d (from time of planting) for the onset of leaf pair 3 and 24 d, 42 d, 65 d, and 96 d respectively for the fourth to seventh pairs. The windowsill on which the plants were grown is south facing and received direct sunlight. Plants for wax extraction were grown in a growth chamber in a light/dark cycle of 12h:12h. The temperature was maintained between 19°C and 21°C. Plants in both locations were fertilized approximately once per month with miracle-grow prepared according to the package directions.

2.2.2: Wax extraction and sample preparation

Leaves for wax analysis were removed as close as possible to the main stem with a razor blade and tweezers. Leaf nodes were counted from the bottom of the plant. Lipid soluble waxes were obtained by immersing the leaves in chloroform for 2 x 30 seconds, which has been shown in previous experiments to remove all extractable waxes. Extracts for each leaf were pooled, and the solvent evaporated under a stream of nitrogen gas. A measured amount of tetracosane (in $CHCl_3$) was added as an internal standard. Dried wax samples were derivatized in N, O bis-trimethylsilyl triflouroacetate (BSTFA) in the

presence of pyridine (volume ratio 1:1) for 45 minutes at 70°C to protect hydroxyl groups as their TMS ethers and prevent the sample from adhering to the gas chromatograph (GC) column. Samples were dried under nitrogen before dissolving in chloroform for chemical analysis.

2.2.3: GC-FID, GC-MS analysis

Chemical analysis was carried out on two gas chromatograph machines equipped with different detection systems. Quantification was accomplished on a GC with flame ionization detector (GC-FID) by comparison with the internal standard. A combination of this information with the area of the extracted leaf (calculated by analysis of a digital photograph with Image J software[5]) allowed calculation of wax amount in $\mu g/cm^2$. Compound identification was carried out on an identical GC machine attached to a quadrupole mass spectrometer (GC-MS) by comparison of mass spectra with a spectral library of known compounds.

The GC-FID instrument used was a 6890N Network GC system (Agilent, USA) equipped with an FID detector. The column used was an HP-1 Methyl Siloxane capillary (Agilent), length 30.0 m, inner diameter 320 μm with a 1.00 μm film thickness. 1 μL of sample was injected and carried by hydrogen gas at a constant flow rate of 2.0 mL/min. The oven program was as follows: 2 minutes at 50˚C followed by a 40˚C/min ramp to 200˚C. The temperature was held at 200˚C for 2 minutes, and then a ramp of 3˚C/min to 320˚C took place. Finally, the oven temperature was held at 320˚C for 30 min. The conditions for analysis by GC-MS were identical except that helium at 2.0 mL/min was used as the carrier gas.

2.2.4: HPLC purification and NMR analysis

The identity of the major triterpenoid component was confirmed by NMR analysis. Approximately 40 leaves were extracted with chloroform and the triterpenoid fraction (Rf ~ 0.3) isolated by preparative thin layer chromatography (2.0mm silica, 99 $CHCl_3$:1EtOH).

The triterpenoid fraction was further separated by preparative HPLC. The HPLC system used was composed of a P680 HPLC Pump and an ASI-100 Automated Sample

Injector (Dionex, Sunnyvale, CA, USA), connected to a Symmetry C_{18} column (Waters, Milford, MA, USA) (5 μm, 4.6 × 250 mm), kept at 30˚C in a Dionex LC30 Chromatography oven, and to a Dionex PDA-100 Photodiode Array Detector. Fraction collection was done with an FC204 fraction collector (Gilson, Middleton, WI, USA). The HPLC instrument was fully controlled by the Chromeleon version 6.40 software from Dionex. Elution was performed using mobile phase A (water with 0.01% phosphoric acid) and mobile phase B (acetonitrile with 0.01% phosphoric acid), with a gradient system as described in Mathe et al.[6] The flow rate was 1 mL/min and detection was at 210 nm. Under these conditions, glutinol eluted at ~60 min.

Fractions from successive runs were pooled and sufficient material obtained to perform ^{1}H-NMR analysis of the major component. NMR analysis was performed on a Bruker 600MHz machine by staff at the UBC NMR facility.

2.3: Results

Leaf measurement was done to establish a time course for leaf growth which could be correlated with wax extraction and eventually enzyme characterization data. The first two leaf pairs emerged in the plantlet stage, were small, and had entire leaf margins. The third and higher pairs had serrated leaf margins and grew larger than the first two pairs. Plantlets grew in the notches of the third and higher leaf pairs and could be easily detached. The third leaf pair was chosen for further analysis due to its resemblance to the upper leaves and its slow growth, which allowed for chemical analysis at several time points during leaf development.

Because measurement of leaf area by flattening and photographing the leaves was destructive, it was necessary to find another way to gauge leaf growth. Leaf shape remained relatively constant throughout development and preliminary measurements (data not shown) have established that leaf length was proportional to area and thus could be used to measure leaf growth.

Prior analysis of mature leaves from other specimens of *K. daigremontiana* has shown that the size of mature leaves is variable, with some reaching a length of 20 to 30 cm. This experiment measured leaves up to and including the seventh leaf pair which gave examples of the growth of both upper and lower leaves. Higher leaves on *K.*

daigremontiana plants reach various sizes, introducing variability and ambiguity. Older leaves are also often partially covered with lesions, which have an unknown effect on their growth.

Results of the leaf measurement experiment are presented followed by chemical analysis from the wax extraction time course.

2.3.1: Leaf growth

Growth slowed for the seventh leaf pair at about 40 days (fig. 2.1). The slow down in growth was later for the sixth and fifth leaf pairs (50 and 60 days respectively) and around 60 days for the lower leaves as well. Leaf length at maturity was greater for each higher leaf pair, and the change in growth rate more dramatic and easier to define.

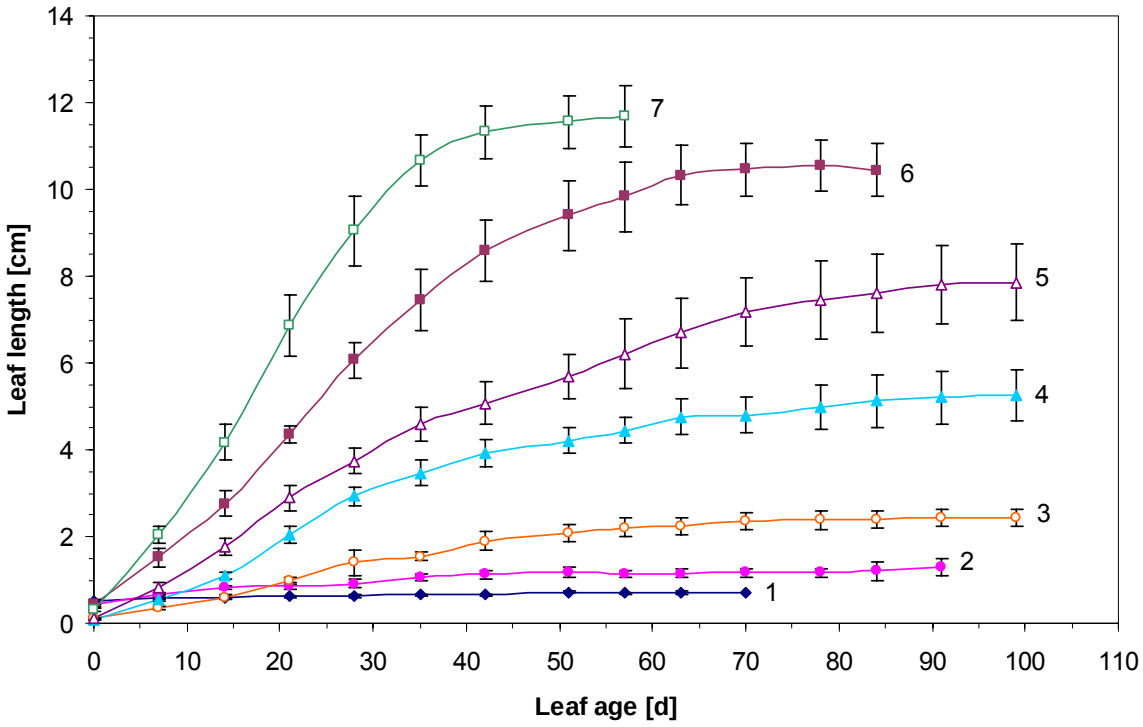

Figure 2.1: Leaf length for different leaf pairs: Leaf length was measured for leaves on the first seven nodes (as counted from the bottom of the plant) for 4 to 8 parallels for each time point. Error bars show standard error. On first leaf pair, error bars are too small to be seen. Measurement on first leaf pair ended early due to loss of leaves

Closer examination of leaf growth for the third leaf pair only (fig. 2.2) reveals that it also stops after ~60 days. These leaves were analyzed after removal from the plant for chemical analysis, allowing leaf area to be measured as well. Comparison of leaf length and area reveals that for the third leaf pair, length can be used to calculate area by the relationship $A = 0.75 \times L^2$. Prior analysis of other leaves (data not shown) had shown similar patterns but with different proportionality constants. This lends further support to the use of leaf length in place of direct area measurement.

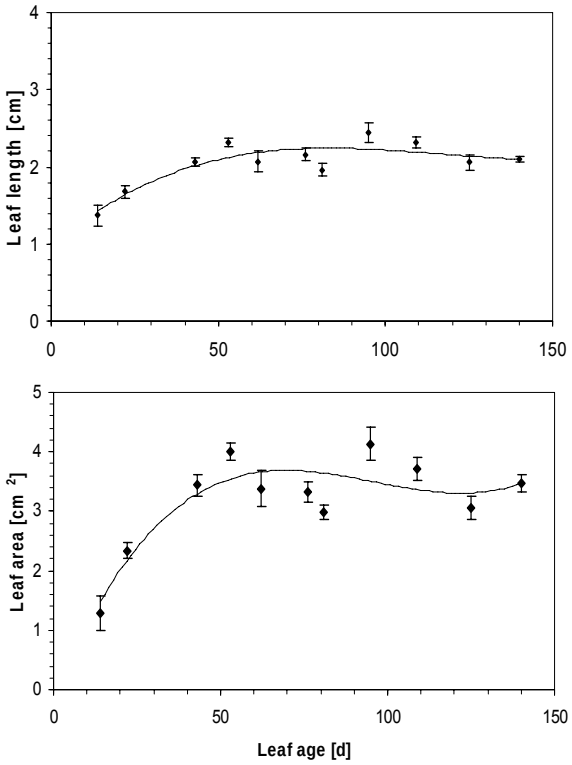

Figure 2.2: Leaf growth for third leaf pair: Leaf length (above) and area (below) were measured for all leaves collected for extraction (n = 3-7, ± standard error)

2.3.2: Chemical Analysis

Wax composition data is first presented for total wax and then broken down to show the different compound classes and their constituents. All extractions were done on the third leaf pair unless otherwise noted.

2.3.2.1: Total Wax

Wax coverage increased for the first 60 d of leaf growth after which there was a brief plateau followed by a further increase (fig.2.3). The cuticular wax coverage reached

a level between 15 - 20µg/cm^2 after about 100 d. Apparent differences in wax coverage after 100 d appear to be the result of biological variability between leaves. There was no statistically significant difference between the wax coverage at 109 d 140 d (2 tailed t-test, $p < 0.05$). Analysis of other mature leaves (data not shown) confirmed the fact that wax coverage of mature leaves is variable, with values ranging from 13 - 30µg/cm^2.

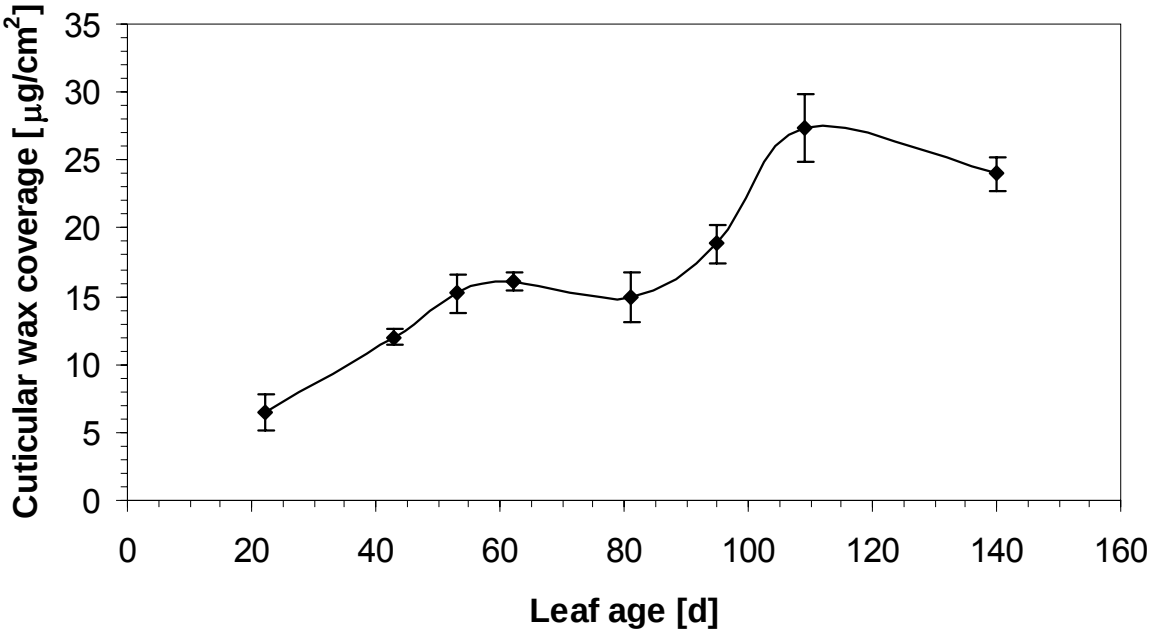

Figure 2.3: Cuticular wax coverage per unit area: Analyses of leaves from third node were carried out on three to six parallels for each time point, error bars show the standard error

2.3.2.2: Wax composition

The relative composition of *K. daigremontiana* wax changed substantially over time (fig.2.4). In young leaves, triterpenoids made up almost 80% of the total wax and VLCFA derivatives made up only a 20%. Over time, the relative proportion of VLCFA derivatives within the wax increased and by day 140 constituted about 34% of the total wax, while triterpenoids contributed less than 50%.

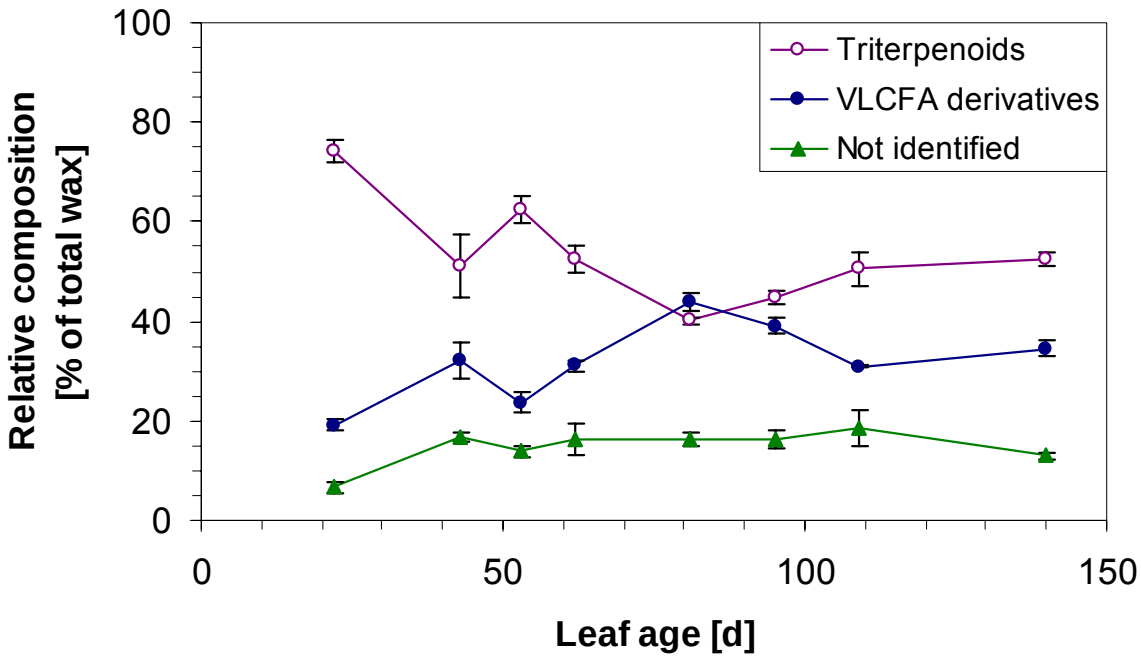

Figure 2.4: Relative wax composition by compound class: Analyses of leaves from third node were carried out on three to six parallels for each time point, error bars show standard error

2.3.2.3: VLCFA and derivatives

The VLCFA derivatives identified in *K. daigremontiana* leaf waxes were unbranched, fully saturated fatty acids, aldehydes, alkanes, primary alcohols and alkyl esters. The composition of VLCFA derivatives in the wax of leaves on the third node did not change significantly over the course of leaf development, containing approximately 70% alkanes and 5 – 15% of each of the other compound classes (Fig. 2.5).

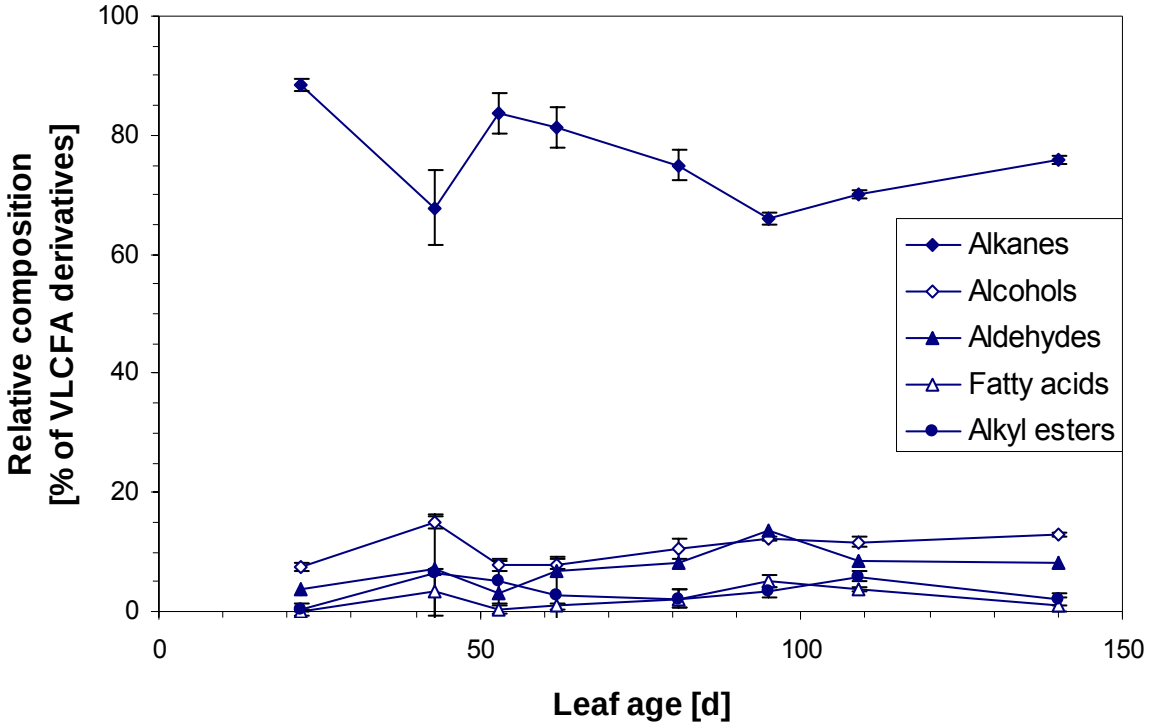

Figure 2.5: Relative composition of VLCFA derivatives: Analyses of leaves from third node were carried out on three to six parallels for each time point, error bars show standard error

The homologous series of alkanes was dominated by compounds with odd carbon numbers, whereas the other VLCFA derivative classes contained mostly even-numbered homologues (Fig. 2.6). The acids, aldehydes and alcohols had similar chain length distributions with a prevalence of C32 and C34 compounds. Esters with overall chain lengths of C48 and C50 were prevalent, and were found to contain C32 and C34 alcohols linked to C16 acid. Some less abundant esters with extreme chain lengths (C42, C44, and C52) could be detected only in the wax of nearly mature leaves, substantially broadening the chain length distribution. In contrast, the homologous series of alkanes was fairly narrow, being dominated by the C33 compound tritriacontane that accounted for approximately 80% of the fraction.

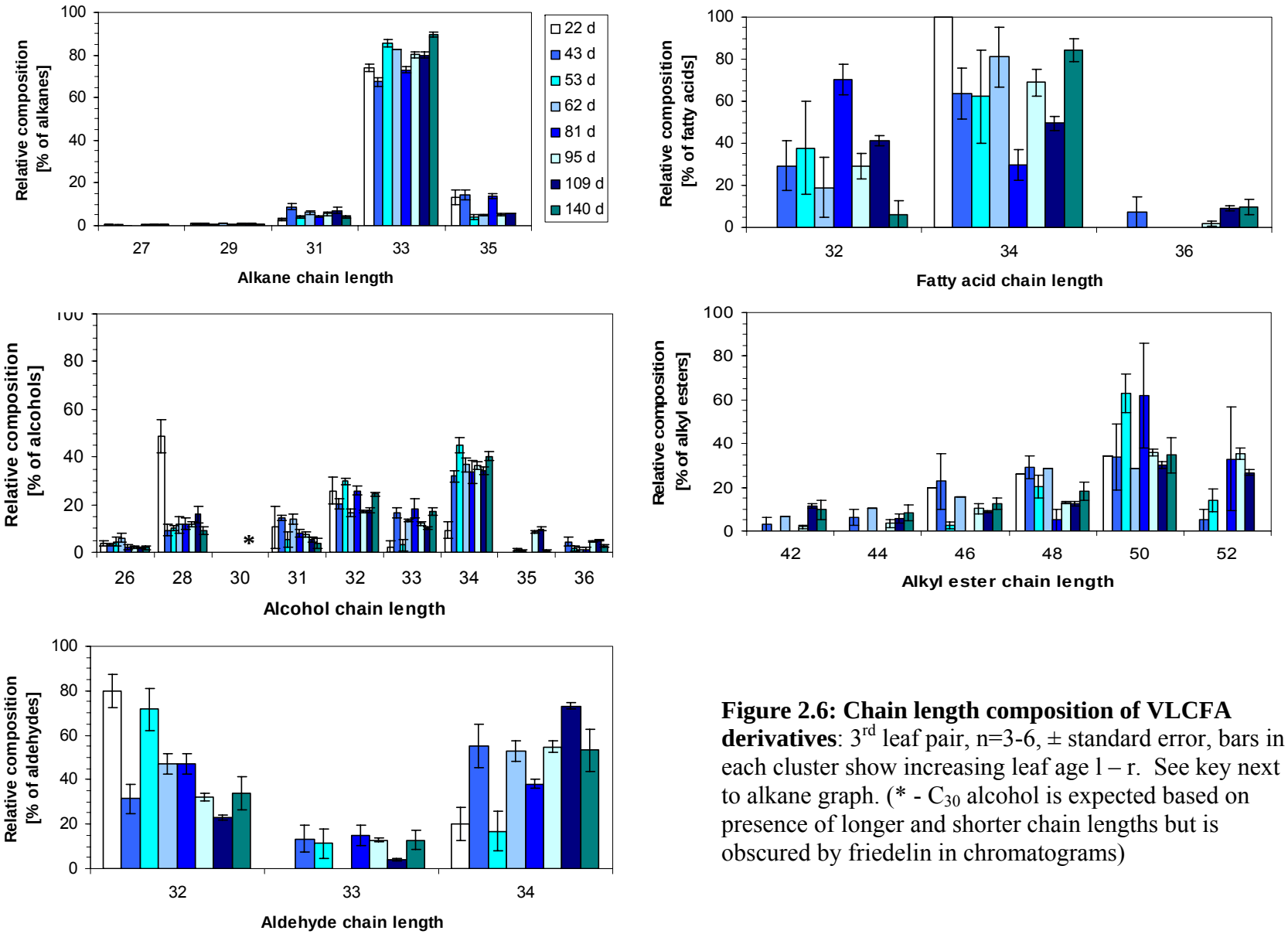

Figure 2.6: Chain length composition of VLCFA derivatives: 3rd leaf pair, n=3-6, ± standard error, bars in each cluster show increasing leaf age l – r. See key next to alkane graph. (* - C_{30} alcohol is expected based on presence of longer and shorter chain lengths but is obscured by friedelin in chromatograms)

2.3.2.4: Triterpenoid composition

A total of eight different triterpenoids were identified in the cuticular wax of *K. daigremontiana*, some in trace amounts and only sporadically. Authentic standards were available for positive identification by co-injection on GC and MS comparison for some of the triterpenoids including β-amyrin, uvaol, and friedelin. Other standards (such as epifriedelanol) had been made in the past by simple transformations of the appropriate standards, providing reliable references for the MS library which were confirmed by comparison with published values. A sample of the type of mass-spectral comparisons that were carried out during compound identification is shown in figure 2.7.

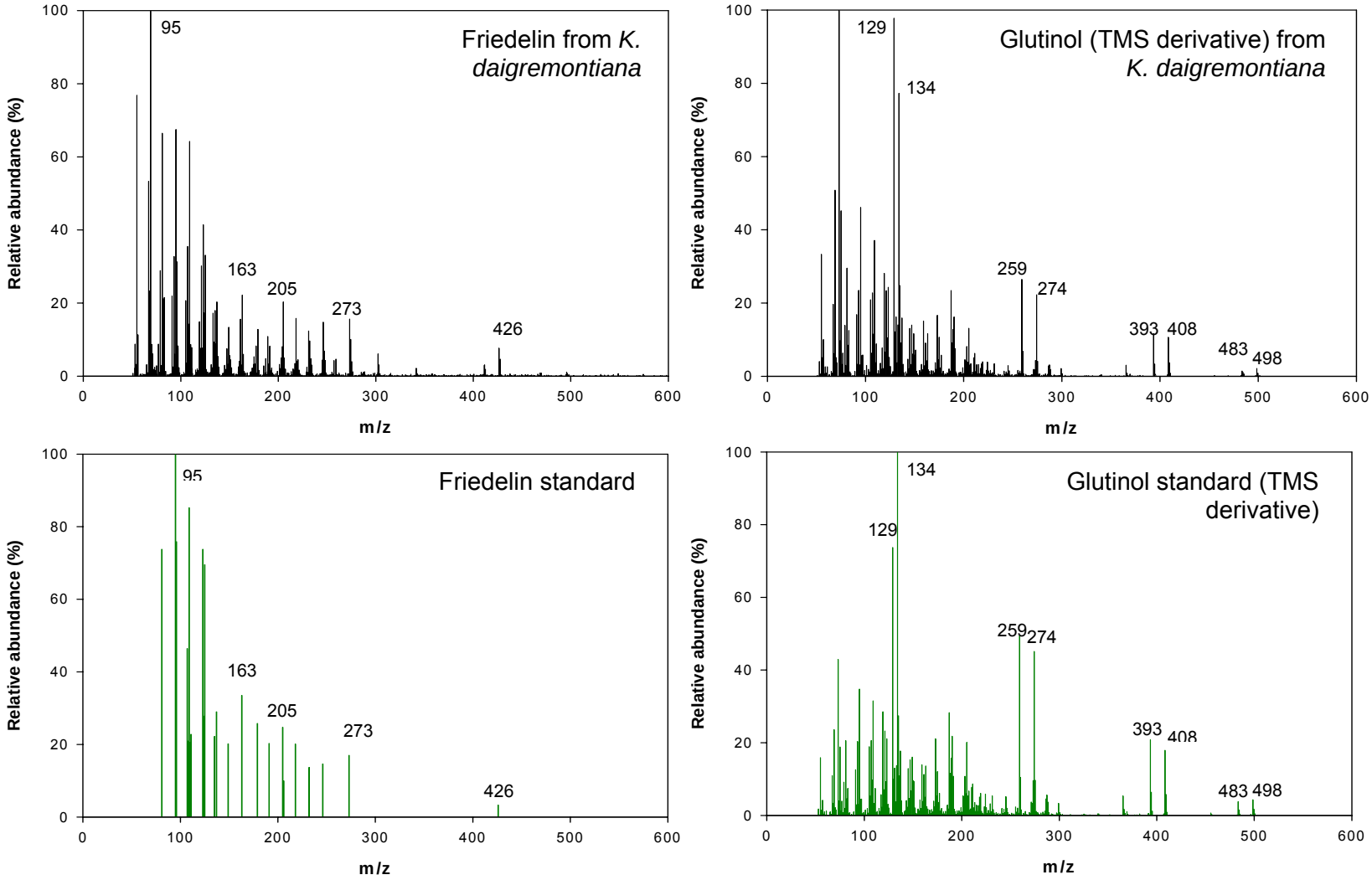

Figure 2.7: Samples of mass-spectral comparisons: Mass-spectra of two compounds found in *K. daigremontiana* (black) and mass-spectra of authentic standards (green). Comparison of mass-spectra enabled compound identification.

Glutinol standard was not readily available, so suspected glutinol had been previously isolated from *K. daigremontiana*, derivatized, and its identity confirmed by comparison with literature values for mass-spectra of its acetates and TMS ethers. To further confirm the identity of glutinol, NMR analysis was performed on the extracted and isolated major triterpenoid peak from mature leaves of *K. daigremontiana*. This compound was subjected to ^{1}H-NMR analysis and was confirmed to be glutinol by comparison with literature values[1, 7] (table 2.1).

Table 2.1: ^{1}H-NMR analysis of glutinol by comparison with published values: NMR analysis was performed on a 600 MHz Bruker spectrometer, numbering refers to glutinol numbering as in fig. 2.8, literature references[1,7] confirm suspected glutinol standard as authentic.

^{1}H – NMR chemical shifts (δ in ppm)		
van Maarseveen 2008	**Gaind 1976**	**Akihisa 1992**
0.86 – 3H, s	0.85 – 3H, s	0.85 – 3H, s, H-25
0.95 – 3H, s	0.94 – 3H, s	0.95 – 3H, s, H-29
1.00 – 3H, s	0.99 – 3H, s	0.99 – 3H, s, H-30
1.01 – 3H, s	1.00 – 3H, s	1.00 – 3H, s, H-27
1.05 – 3H, s	1.05 – 3H, s	1.04 – 3H, s, H-23
1.10 – 3H, s	1.09 – 3H, s	1.09 – 3H, s, H-26
1.15 – 3H, s	1.14 – 3H, s	1.14 – 3H, s, H-24
1.17 – 3H, s	1.17 – 3H, s	1.16 – 3H, s, H-28
3.48 – 1H, br s	3.46 – 1H, br s	3.49 – 1H, m, H -3α
5.64 – 1 H, br d	5.62 – 1H, br s	5.63 – 1H, br d, H-6

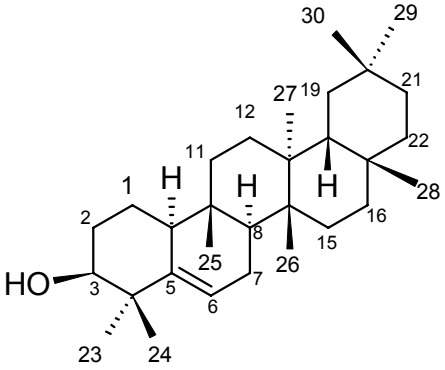

Figure 2.8: Structure of glutinol: alternate name 3-β-hydroxy-glutin-5-ene, shown with carbon numbering

Although neither of these publications reported or assigned the chemical shifts of the methylene or bridgehead hydrogens, signals were observed between 0.90 and 2.05 but were impossible to assign. Perhaps this is why previous publications did not report or assign these signals. Co-injection and comparisons confirmed that the compound previously identified by mass spectral comparisons was indeed glutinol. In light of this, the identities of previously prepared derivatives of this standard (glutanol and glutinol acetate) could be verified.

Early in leaf development, glutinol made up about 70% of the triterpenoid fraction of the wax (fig. 2.9). The relative concentration of glutinol decreased to between 40% and 50% after about 100 days. Friedelin comprised about 20% of the triterpenoids throughout leaf development, while germanicol contributed about 10% to the total.

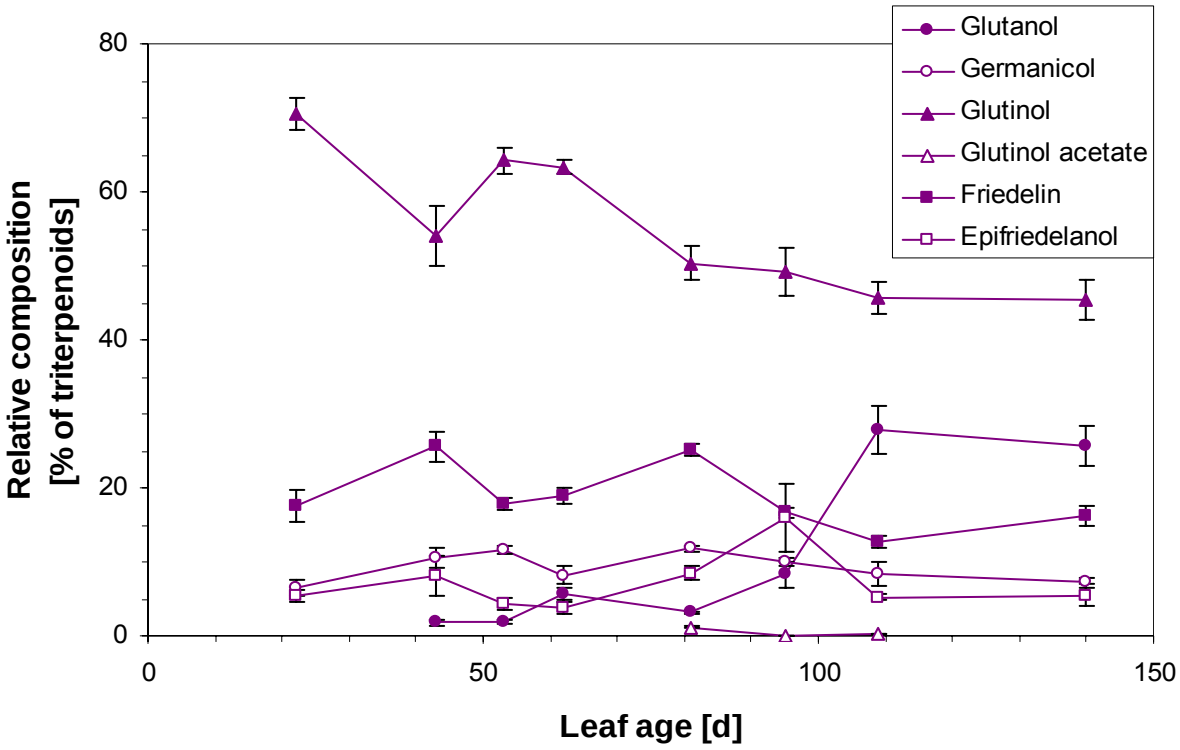

Figure 2.9: Relative composition of triterpenoid fraction of cuticular wax: Analyses of leaves from third node were carried out on three to six parallels for each time point, error bars show standard error

Epifriedelanol, the reduced form of friedelin, made up less than 20 percent of the total triterpenoid content throughout leaf development. Glutanol, the reduced form of glutinol, increased from less than 10% of the triterpenoid concentration to over 20% between 100 and 110 days leaf age (fig.2.9). Glutinol acetate, β-amyrin, and uvaol were detected in trace amounts sporadically throughout the course of the experiment. Insufficient amounts of β-amyrin and uvaol were detected for reliable quantification.

2.4: Discussion

Leaf growth is clearly related to the leaf's position on the plant, with the lower leaves growing more slowly and reaching a smaller size when mature than upper leaves. Because measurements were only made of the first seven pairs of leaves, it is unclear how far such a trend can be extrapolated, but observations of mature plants indicate that large leaves higher on the plant reach a maximum size of 20 to 30 cm but not longer, so the trend of higher leaves being larger cannot be extended indefinitely.

Comparing cuticular wax accumulation with leaf growth data, the initial slow down in wax accumulation around 60 d (fig. 2.3) corresponds well with a slower growth rate at about the same time (fig. 2.1). Based on these observations, wax production overcompensates for leaf growth during the first 50 to 60 d of leaf development, after which leaf growth and wax production both slow down and eventually stop. The processes are thus well synchronized. Variation in wax amount per unit area observed later in leaf development (95 d and later) may be due to biological variation between leaves rather than a second increase in wax coverage and should not be over-interpreted.

Throughout leaf development, the relative composition of VLCFA derivatives remains relatively constant with respect to individual compound classes (fig. 2.5) and the chain length distribution within each compound class (fig.2.6). The overall predominance of the C_{34} homologues of fatty acids and aldehydes corresponded to that in free and esterified alcohols, reflecting the biosynthetic relationships between these compound classes. Similarly, the predominance of C_{33} alkane in *K. daigremontiana* leaf wax confirms the conclusion, drawn from wax chemical and biochemical investigations of other species, that cuticular wax hydrocarbons are formed by loss of a C_1 unit from corresponding acid or aldehyde precursors.

The predominant C_{34}/C_{33} homologs in *K. daigremontiana* wax are longer than those in wax mixtures of most plant species investigated to date. Only relatively few species, for example sesame and soybean, had been found to have waxes dominated by C_{32}/C_{33} and C_{32}/C_{31} chainlengths, respectively.[8, 9] Interestingly, a published analysis of lipid soluble extracts from *K. pinnata* reported the presence of C_{31} and C_{33} n-alkanes as well as alcohols with chain lengths up to C_{36}.[10]

Getting to these extremely long chain lengths requires more than the usual number of rounds of elongation and reduction. Within the FAE complex, the condensing enzyme responsible for the first step of the elongation-reduction cycle, is known to show chain length and product specificity.[11] The FAE complex of *K. daigremontiana* has not been characterized and thus it is not known whether there is a specialized condensing enzyme in this species to deal with the extra long chains, or if the enzyme simply has broader chain length specificity and so can continue with further rounds of elongation.

In the alcohols (fig. 2.6), the distinction between the major (even chain length) and minor (odd chain length) components is less clear than in the other compound classes. It appears that in *K. daigremontiana,* and perhaps other plants with longer than average chain lengths in their cuticles, there is less distinction between the major and minor chain lengths than in the case of "normal" chain lengths. This may be due to the elongation cycle starting with a three-carbon unit which is extended by two-carbons in each cycle, yielding an elongation product with an odd numbered carbon chain length.

In absolute terms, the amount of triterpenoids per unit area increases from ca. 5 $\mu g/cm^2$ to ca. 12 $\mu g/cm^2$ between 22 d and 140 d. During the same time interval, the absolute amount of VLCFA derivatives in the wax increased by a factor of 8 (from ~1 to ~8 $\mu g/cm^2$). Therefore, while triterpenoids are the predominant compound class throughout the course of leaf development, it is obvious that the relative amount of triterpenoids decreases over the course of leaf development as the relative abundance of VLCFA derivatives (particularly alkanes) increases. A similar change in relative abundance has been observed in other experiments with cherries and tomatoes [3, 4] suggesting a common biological phenomenon.

Analysis of the fifth leaf pair of *K. daigremontiana* (Han, H., personal communication) confirmed the pattern of decreasing relative triterpenoid concentration

coincident with an increase in the relative amount of VLCFA derivatives. In these leaves VLCFA derivatives increased from 22 ± 4% to 46 ± 1% of the total wax between 4 and ~30 weeks leaf age. During the same time period, the relative amount of triterpenoids in the cuticular wax decreased from 69 ± 4% to 33 ± 2% of the total.

The biological function(s) of relatively higher triterpenoid concentration in young developing cuticles is not known. However, some triterpenoids and triterpenoid saponins have been implicated as insect antifeedants [12, 13] and many triterpenoids have biological activity.[14, 15] Assessing the biological role (if any) of triterpenoids in *K. daigremontiana* would require either silencing or overexpression of a triterpenoid synthase as well as an appropriate pathogen/plant system.

Confirmation of the structure and identity of glutinol was made possible by analysis of both mass-spectral and NMR data. Comparison of published ^{1}H-NMR data with ^{1}H-NMR spectra of suspected glutinol from *K. daigremontiana* showed close agreement in both cases (table 2.1). The fact that neither publication reported or assigned the methylene or bridgehead hydrogens is probably due to the fact that the region of the spectrum from ~ 1 – 2 ppm is very crowded and coupling patterns are complex, leading to overlapping multiplets with coupling constants that are in many cases impossible to establish. The high number of unique hydrogen environments makes interpretation of the spectrum challenging, although it is interesting that neither prior publication chose to report these chemical shifts, even if they were not assigned. Even with confirmatory evidence from NMR pointing to this compound being glutinol, it is possible that another triterpenoid structure may give similar or identical signals to these, even if for different hydrogens. However, another structure with a double bond located in a position other than 5-6 (fig. 2.8) would not give a confirmatory mass spectrum. The characteristic peaks at m/z = 259 and 274 in the MS (fig. 2.7) are due to a Retro-Diels-Alder fragmentation of the B ring and subsequent loss of a methyl group. Alternative placement of the double bond to form another deprotonation product along this pathway would not give these fragments when ionized. Thus, the combination of mass-spectral and NMR evidence conclusively confirms the identity of glutinol.

Glutinol and friedelin, the two major triterpenoid products in *K. daigremontiana* wax, are closely related in terms of their proposed biosynthetic mechanism. Based on

their structures, it is possible that they could be produced by a single enzyme, which would be a multifunctional oxidosqualene cyclase (OSC), potentially producing other products. However, the ratio of glutinol to friedelin fluctuates between 4:1 and 2:1 over the course of the study, implying the involvement of more than one enzyme. Previous work on the characterization of triterpenoid synthases has identified and characterized a variety of both multifunctional and single product enzymes.[16] Therefore, these two (or more) enzymes may produce either single or multiple products, which may then be modified by acetylation or reduction. Taking as a group the glutinol-like compounds (glutinol, glutinol acetate and glutanol) and comparing these to the friedelin-like compounds (friedelin and epifriedelanol), we see that the ratio of glutinol-like to friedelin-like compounds fluctuates between 3:1 and 7:4, lending further support for the involvement of more than one enzyme. This is not unlikely, since several putative OSCs have been identified in *K. daigremontiana* based on sequence homology.

Different rates of cuticular accumulation for different triterpenoids may be the result of gene or enzyme expression or export to the cuticle. In order to address these questions, genes must be cloned and characterized and this data ultimately compared with product accumulation results. Based on the product accumulation curves described here, the time points with highest enzyme/gene activity can now be selected. Further work to characterize these genes is ongoing.

2.5: Conclusions:

Investigation of the cuticular wax composition of *K. daigremontiana* during leaf development has revealed patterns in the accumulation of triterpenoids and VLCFA components within the wax. Based on these experiments, it can be concluded that triterpenoid biosynthesis is most active early in leaf development while the synthesis and export of VLCFA components continues and increases throughout leaf growth. Differences in the accumulation patterns of various triterpenoids give support to the hypothesis that more than one triterpenoid synthase is active in *K. daigremontiana*.

References

(1) Gaind, K. N.; Singla, A. K.; Boar, R. B.; Copsey, D. B. *Phytochemistry* **1976**, *15*, 1999-2000.

(2) Siems, K.; Jas, G.; Arriagaginer, E. J.; Wollenweber, E.; Dorr, M. *Z. Naturforsch., C: J. Biosci.* **1995**, *50*, 451-454.

(3) Peschel, S.; Franke, R.; Schreiber, L.; Knoche, M. *Phytochemistry* **2007**, *68*, 1017-1025.

(4) Leide, J.; Hildebrandt, U.; Reussing, K.; Riederer, M.; Vogg, G. *Plant Physiol.* **2007**, *144*, 1667-1679.

(5) Rasband, W. S. **1997-2007**, *1.38X*.

(6) Mathe, C.; Culioli, G.; Archier, P.; Vieillescazes, C. *J. Chromatogr. A.* **2004**, *1023*, 277-285.

(7) Akihisa, T.; Yamamoto, K.; Tamura, T.; Kimura, Y.; Iida, T.; Nambara, T. *Chem. Pharm. Bull.* **1992**, *40*, 789-798.

(8) Kim, K. S.; Park, S. H.; Jenks, M. A. *J. Plant Physiol.* **2007**, *164*, 1134-1143.

(9) Kim, K. S.; Park, S. H.; Kim, D. K.; Jenks, M. A. *Int. J. Plant Sci.* **2007**, *168*, 307-316.

(10) Gaind, K. N.; Gupta, R. L. *Planta Med.* **1974**, *25*, 193-197.

(11) Millar, A. A.; Kunst, L. *Plant J.l* **1997**, *12*, 121-131.

(12) Eigenbrode, S. D.; Pillai, S. K. *J. Chem. Ecol.* **1998**, *24*, 1611-1627.

(13) Shinoda, T.; Nagao, T.; Nakayama, M.; Serizawa, H.; Koshioka, M.; Okabe, H.; Kawai, A. *J. Chem. Ecol.* **2002**, *28*, 587-599.

(14) Mahato, S. B.; Sen, S. *Phytochemistry* **1997**, *44*, 1185-1236.

(15) Sun, H.; Fang, W. S.; Wang, W. Z.; Hu, C. *Bot. Studies* **2006**, *47*, 339-368.

(16) Phillips, D. R.; Rasbery, J. M.; Bartel, B.; Matsuda, S. P. T. *Curr. Opin. Plant Biol.* **2006**, *9*, 305-314.

Chapter 3: Epicuticular and intracuticular wax composition of *Kalanchoe diagremontiana* leaves

3.1: Introduction

The cuticle provides a barrier to non-stomatal water loss for most aerial portions of terrestrial plants, but has other properties as well. These include the ability to attenuate UV radiation,[1] self cleaning properties,[2] and the ability to influence the behaviour of insect herbivores through chemical and physical interactions.[3-5] In addition to the more general properties, some plants have specialized cuticles on some organs which fulfill specific needs. For example, some plants use specialized leaves which form a trap to catch insects. A slippery cuticle prevents the insects from climbing out.[6, 7] Other plants' stems are covered with epicuticular wax crystals which control insect access to upper regions of the plant.[8]

In order to be available for interaction with the outside world, compounds must be present in either the epicuticular wax film or epicuticular wax crystals, which protrude from the wax film in some organs and species. The chemical composition and structure of these crystals can be studied by selective analysis and scanning electron microscopy (SEM) respectively. Previous reviews have summarized some of the established correlations between epicuticular wax composition and crystal form.[9, 10] Some conclusions regarding the composition of crystals have been confirmed by growing crystals of known composition in vitro which resemble those seen on plant surfaces.[11, 12] A summary of some known crystal forms and their compositions was presented in chapter 1 (pg. 3-4) and will not be repeated at this time.

Despite all that is known about epicuticular wax crystals, many questions remain. Crystals are often observed when there is a high concentration of a single compound in the epicuticular wax, implying that there is a threshold which must be achieved before crystals can form. Whether the threshold for crystal formation is in absolute amount of a compound (in $\mu g/cm^2$) or its relative concentration with respect to other wax components is unclear. Comprehensive chemical and microscopic analysis of additional species would add to our understanding of crystal composition and formation. Essential for an understanding of wax crystals and how they form is a complete picture of the epicuticular and intracuticular wax composition of a species.

In triterpenoid containing species, gradients tend to be seen with triterpenoids being found primarily in the intracuticular wax while alkanes and other VLCFA derivatives are found in the epicuticular wax layer.[13-15] This gradient may be due to the polarity and/or geometry of polycyclic triterpenoids and the fact that they may interfere with the packing pattern of the very long chain wax components and therefore may not diffuse easily through the cuticular wax. However, in some cases such as some *Macaranga* species, triterpenoids are found in the epicuticular wax crystals, meaning that they must be present in the epicuticular wax.[8] Because of the variety of triterpenoid distributions in different cuticles, it is impossible to draw a simple conclusion about how and why gradients are established between the epicuticular and intracuticular wax layers.

The cuticular wax of *Kalanchoe daigremontiana* has been previously analyzed and found to contain triterpenoids. The high concentrations of friedelin and glutinol, two relatively uncommon triterpenoids are of interest due to the extensive charge migration required before deprotonation (see fig. 1.6). A time course study of *K. daigremontiana* wax composition found a decrease in relative concentration of triterpenoids during leaf ontogenesis (chapter 2). A similar pattern of decreasing triterpenoid abundance had been noted for both developing cherries and tomatoes.[16, 17] Because triterpenoids were more abundant in the young and potentially more vulnerable leaf and fruit cuticles, questions arose about the function of triterpenoids in the cuticular wax, particularly whether they play a role in protecting the developing organ.

Whether triterpenoids serve an ecological function and moderate plant-insect or plant-pathogen interactions depends on where the triterpenoids are found. If triterpenoids are found in the intracuticular wax layer they would not be available for insect or pathogen interactions at the plant's surface. However, if epicuticular triterpenoid crystals are present, they would be available for interactions at the surface and may play some (as yet undefined) ecological role.

In the case of *K. daigremontiana*, the appearance of the adaxial (top) and abaxial (bottom) leaf surfaces gave reason to suspect a difference in composition, with the abaxial surface being noticeably more glaucous than the adaxial surface. Since a glaucous surface usually indicates the presence of epicuticular wax crystals, there was reason to investigate further and determine the composition of any such crystals.

Because of the nature of epicuticular and intracuticular wax analysis, it is only possible to analyze the composition of one side of the leaf at a time, thus previous data based on the cuticular wax composition of the entire leaf could not be used as a reference. Since differences between adaxial and abaxial wax composition had been previously reported in pea cultivars[18] and nothing was known about the composition of cuticular waxes on the adaxial and abaxial surfaces of *K. daigremontiana* leaves, it was decided that both surfaces would be analyzed.

Knowing that *K. daigremontiana* wax contained a high proportion of triterpenoids as well as epicuticular wax crystals raised questions. Are the crystals made of triterpenoids or of other wax components? What drives wax crystal formation? What type of gradient is established in the cuticular wax of *K. daigremontiana*? In order to address these large questions, goals of the research were established. These were:

a) To compare and contrast the chemical composition of the adaxial and abaxial epicuticular waxes

b) To compare and contrast the chemical composition of the epicuticular and intracuticular waxes on both leaf surfaces

c) To investigate the physical structure of the adaxial and abaxial epicuticular waxes.

3.2: Materials and Methods

Relevant details regarding plant growth and maintenance, physical and chemical removal of waxes, and subsequent chemical analyses are presented as well as details relating to SEM analysis.

3.2.1: Plant material

Plants for this experiment were grown in ambient conditions in the laboratory. They were exposed to sun through a South facing window, watered approximately every 3-4 days and fertilized approximately once per month. Mature leaves were removed close to the stem with tweezers and a razor blade for wax analysis.

3.2.2: Isolation of waxes

Epicuticular wax was removed by adhesion with gum arabic.[13] Circles of known area were marked on the leaves and then painted with a gum arabic solution (concentration 1.5g/ml). After drying, the gum arabic layer was peeled off. Waxes were removed from dried gum arabic by extraction with chloroform. At this time a measured amount of tetracosane standard was added and solvents were evaporated under nitrogen gas. To determine the effectiveness of the gum arabic peels, a separate experiment was performed in which a dedicated portion of the adaxial leaf surface was treated with gum arabic in four successive treatments. Each treatment was carried out as described above. Three parallels of this repeated peel experiment were performed.

After mechanical removal of waxes with gum arabic, the remaining waxes were extracted with chloroform. A glass cylinder was held in place on the leaf's surface while approximately 1.5mL of chloroform was administered. The chloroform was agitated gently with a Pasteur pipette for 30s and removed. This was repeated with fresh chloroform and the extracts thus collected were pooled. Tetracosane standard was added and the solvent evaporated under nitrogen gas. Wax concentrations were measured by combining quantitative information derived from GC-FID analysis with the known area of the opening in the cylinder used in the extractions.

Single extractions of complete adaxial and abaxial wax were performed using the same "cylinder" method, but without prior gum arabic treatment. Chloroform was applied to the leaf surface for two 30 second intervals and a measured amount of tetracosane standard added to the combined extracts which were then dried under a stream of nitrogen gas. In all applications of the cylinder extraction method, samples were discarded if the leaf surface was damaged or if chloroform leaked out under the glass cylinder.

3.2.3: Sample preparation and chemical analysis

Sample preparation, GC-FID, and GC-MS analysis were all carried out using standard procedures as described in chapter 2 (pg. 25-26).

3.2.4: Sample preparation and SEM analysis

Samples for microscopy were prepared by affixing to an adhesive microscope stub and air drying overnight. Samples were then sputter coated with 5 nm of gold (Au) in a Cressington Sputter Coater 208 H (Ted Pella, Inc.,Redding, CA, USA) before viewing under a Hitachi S4700 field emission scanning electron microscope (SEM; NisseiSangyo America, Ltd, Pleasanton, CA, USA) at a 1.5 kV accelerating voltage and a 12 mm working distance.

3.3: Results

The purpose of the present experiment was to analyze the epicuticular and intracuticular wax composition of *K. daigremontiana*, specifically seeking to understand the distribution of triterpenoids within the wax. Experiments were carried out to assess the effectiveness of epicuticular wax peels with gum arabic and chemical analyses of each leaf surface were performed for both the epicuticular and intracuticular layers. A parallel experiment in which the total wax from each leaf surface was removed in a single extraction provided a reference, and SEM analysis complemented the chemical data.

3.3.1: Effectiveness of gum arabic treatment for removal of epicuticular wax

An experiment was performed on the adaxial leaf surface to determine the minimum number of gum arabic treatments required for exhaustive removal of epicuticular wax. In this experiment, the same leaf area was treated with four successive applications of gum arabic solution, each of which was peeled off and analyzed. The wax yields were: 13.5 ± 2.4 μg cm^{-2} for the first treatment, 2.6 ± 1.0 μg cm^{-2} for the second, 1.6 ± 1.0 μg cm^{-2} for the third, and 0.31 ± 0.24 μg cm^{-2} for the fourth treatment (means ± std. dev., fig. 3.1(A)). During this process, a number of samples were damaged. Although nine samples were treated with the first gum arabic treatment, only eight survived. Four survived the second treatment, and only three survived the third and fourth treatments. These data show that over 75% of the epicuticular wax was removed in the first gum arabic treatment. Statistical analysis of the cumulative wax yield obtained by successive treatments (fig. 3.1(B)) revealed no significant difference in the wax yield after the second treatment (2 tailed t-tests, $p>0.05$) with the increase between

first and first + second treatment amounts only barely reaching statistical significance (2 tailed t-test, p=0.04).

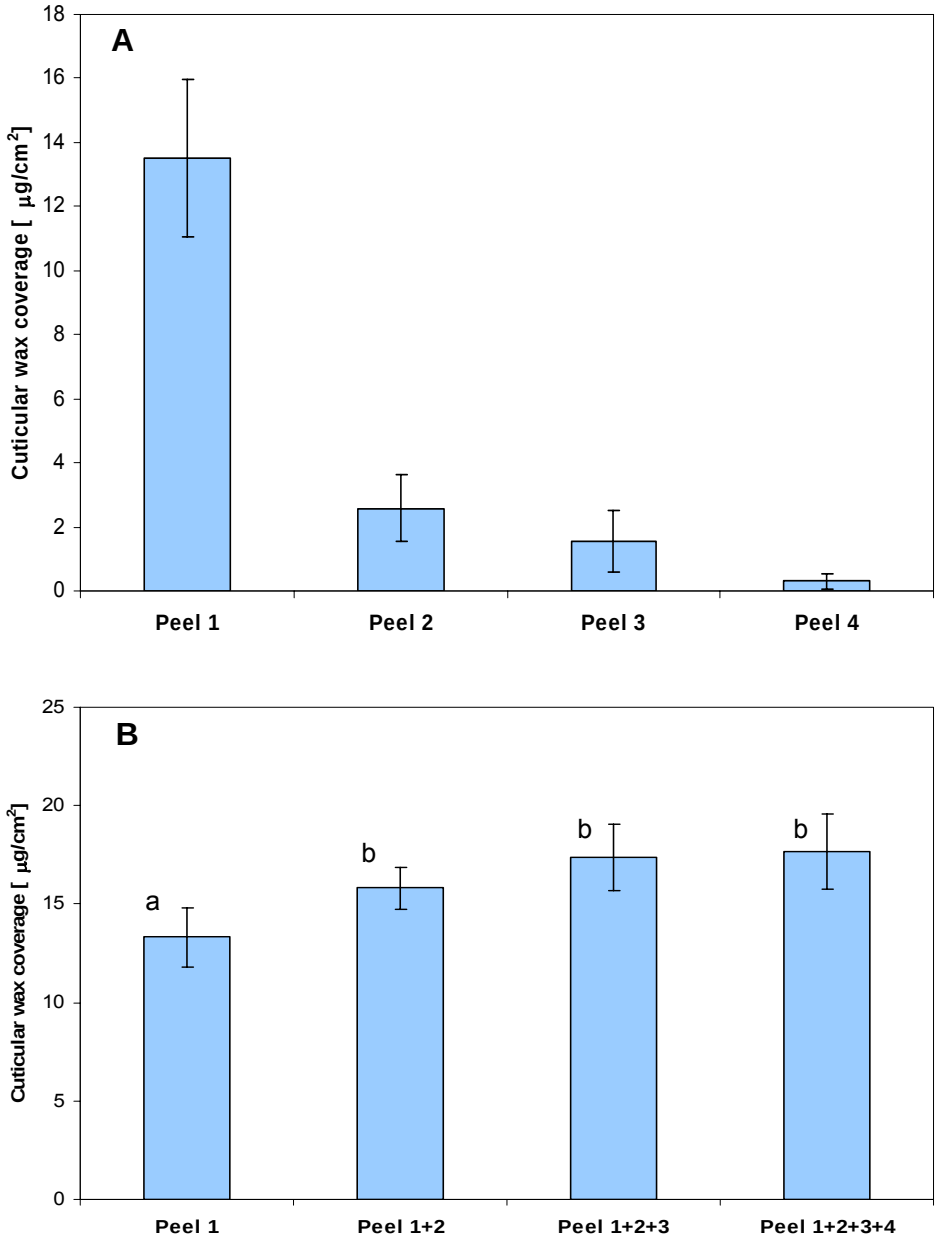

Figure 3.1: Yield of successive physical wax removals by gum arabic: A) wax obtained by successive gum arabic treatments, n= 3-5; B) cumulative wax totals from four successive gum arabic treatments, n=3, different lower case letters indicate statistically significant differences ($p<0.05$); all bars show mean ± standard deviation

In light of this information and the fragility of the leaf material, further analysis was conducted by performing a single gum arabic treatment followed by chloroform extraction to remove the remaining wax. Subsequent results and figures will refer to epicuticular and intracuticular wax for wax obtained by gum arabic (GA) treatment and chloroform ($CHCl_3$) extraction respectively.

3.3.2: Abaxial wax composition

The epicuticular and intracuticular wax coverages were 10 ± 1 µg/cm^2 and 8 ± 2 µg/cm^2, respectively (fig. 3.2) Very long chain compounds were more abundant in the

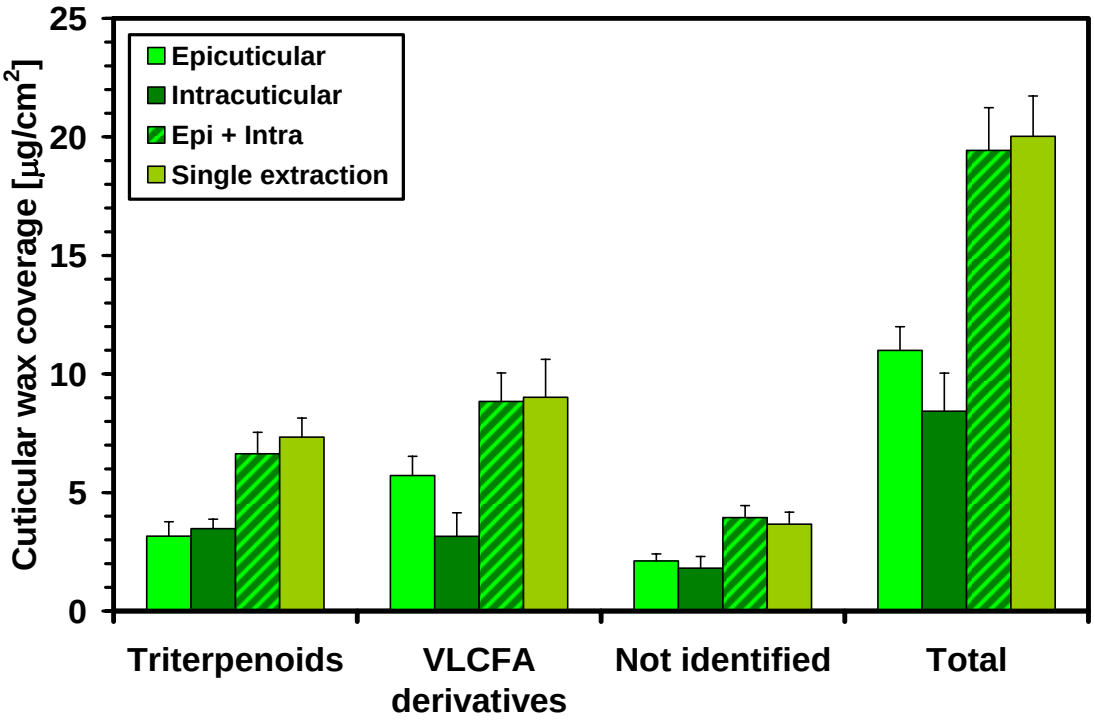

Figure 3.2: Abaxial wax composition by compound class: experiments were performed on five parallels for epicuticular, intracuticular, combined (epi+intra) and single extractions, error bars show std. error

epicuticular layer (5.7 ± 0.8 µg/cm^2) than in the intracuticular layer (3 ± 1 µg/cm^2), while triterpenoids were found in approximately equal amounts in both epicuticular and intracuticular wax (3.2 ± 0.6 µg/cm^2 and 3.5 ± 0.4 µg/cm^2 respectively – all means ± std. error). In relative terms, VLCFA derivatives made up 52 ± 4% of the epicuticular and 34

± 4% of the intracuticular wax while triterpenoids made up 29 ± 5% and 46 ± 5% of the two layers respectively. Approximately 20% of the wax constituents in each the epicuticular and intracuticular layers were unidentified.

Among the VLCFA derivatives, compound classes identified were alkanes, primary alcohols, aldehydes, fatty acids, and alkyl esters. Alkanes were the most abundant compound class (fig. 3.3), making up 33 ± 2% of the epicuticular and 19 ± 1% of the intracuticular waxes. Other VLCFA compound classes each contributed less than 7% to the epicuticular and 6% to the intracuticular layers.

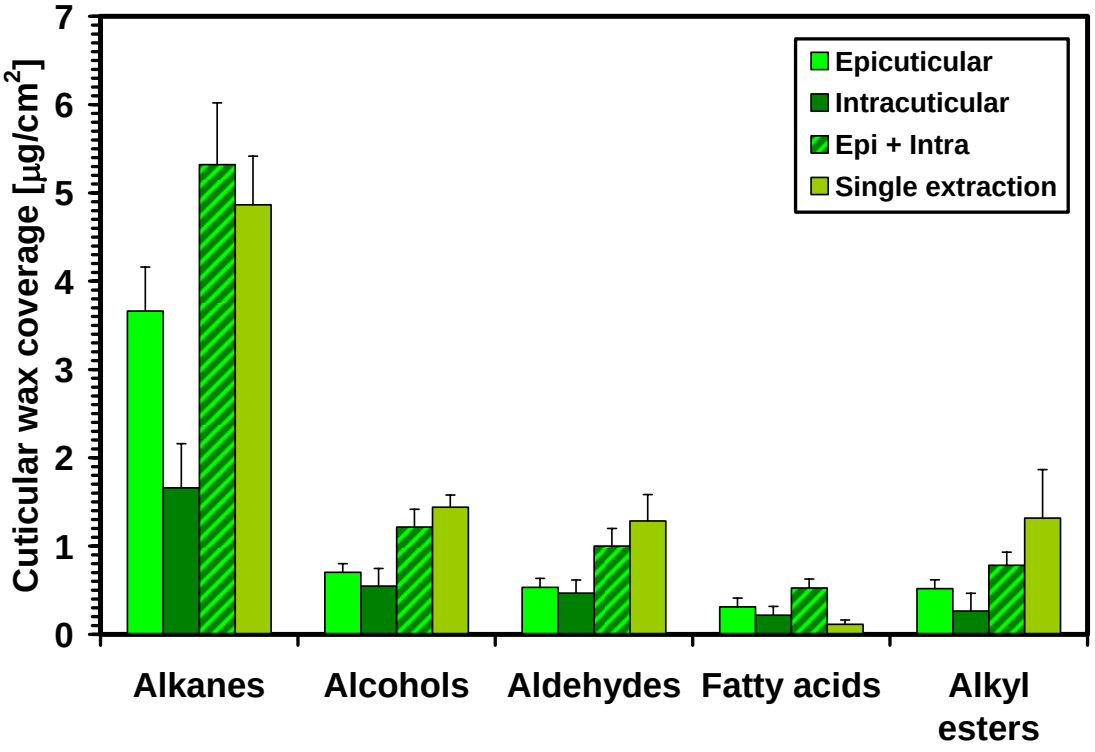

Figure 3.3: Composition of VLCFA derivative classes of abaxial wax: experiments were performed on five parallels for epicuticular, intracuticular, combined (epi+intra) and single extractions. Error bars show standard error

A range of chain lengths from C_{26} to C_{36} was found for most VLCFA derivatives, while in the case of alkyl esters, the chain lengths ranged from C_{42} to C_{52} (table 3.1). In the alkanes, the dominant compound was tritriacontane (C_{33}), and odd chain lengths clearly dominated the chain length distribution. In the aldehydes, fatty acids, and primary alcohols, C_{34} was the dominant chain length and even chain lengths were more abundant

Table 3.1: VLCFA chain length distribution of abaxial wax: Analyses were carried out on five parallels per experiment. Data are shown as mean ± standard error in µg/cm^2 for epicuticular, intracuticular, combined (epi+intra) and single extraction. tr. = trace, n.d. = not detected

	Epicuticular	Intracuticular	Epi + Intra	Single extraction
Alkane chain length				
27	n.d.	n.d.	n.d.	0.008 ± 0.004
29	0.09 ± 0.05	0.02 ± 0.01	0.11 ± 0.05	0.029 ± 0.009
30	n.d.	0.016 ± 0.005	0.016 ± 0.005	n.d.
31	0.4 ± 0.3	0.05 ± 0.01	0.5 ± 0.3	0.14 ± 0.04
33	2.5 ± 0.3	1.4 ± 0.4	3.9 ± 0.5	4.0 ± 0.5
34	0.18 ± 0.03	n.d.	n.d.	0.24 ± 0.04
35	0.41 ± 0.08	0.22 ± 0.08	0.63 ± 0.10	0.49 ± 0.03
total alkanes	*3.7 ± 0.5*	*1.7 ± 0.5*	*5.3 ± 0.7*	*4.9 ± 0.5*
Alcohol chain length				
26	0.04 ± 0.03	n.d.	0.04 ± 0.03	tr.
28	0.06 ± 0.02	n.d.	0.06 ± 0.02	0.17 ± 0.01
31	0.07 ± 0.01	0.07 ± 0.03	0.15 ± 0.02	0.13 ± 0.03
32	0.20 ± 0.07	0.10 ± 0.05	0.30 ± 0.06	0.26 ± 0.07
33	0.09 ± 0.03	0.08 ± 0.03	0.17 ± 0.04	0.18 ± 0.03
34	0.20 ± 0.04	0.21 ± 0.06	0.42 ± 0.07	0.56 ± 0.06
35	tr. ± tr.	0.03 ± 0.02	0.04 ± 0.01	0.06 ± 0.03
36	0.026 ± 0.008	0.05 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
total alcohols	*0.7 ± 0.1*	*1 ± 0*	*1.2 ± 0.2*	*1.4 ± 0.1*
Aldehyde chain length				
32	0.10 ± 0.03	0.16 ± 0.05	0.26 ± 0.02	0.32 ± 0.05
33	0.10 ± 0.03	n.d.	0.10 ± 0.03	0.14 ± 0.01
34	0.33 ± 0.09	0.3 ± 0.1	0.6 ± 0.1	0.8 ± 0.3
total aldehydes	*0.5 ± 0.1*	*0.5 ± 0.1*	*1.0 ± 0.2*	*1.3 ± 0.3*
Fatty acid chain length				
32	0.10 ± 0.04	tr.	0.13 ± 0.03	n.d.
34	0.18 ± 0.07	0.19 ± 0.10	0.37 ± 0.08	0.11 ± 0.05
total fatty acids	*0.31 ± 0.09*	*0.2 ± 0.1*	*0.5 ± 0.1*	*0.11 ± 0.05*
Alkyl ester chain length				
42	tr.	tr.	0.07 ± 0.04	n.d.
44	tr.	tr.	0.011 ± 0.007	0.01 ± 0.01
45	n.d.	n.d.	n.d.	tr.
46	0.03 ± 0.01	tr.	0.04 ± 0.01	0.06 ± 0.02
47	n.d.	n.d.	n.d.	tr.
48	0.12 ± 0.04	0.04 ± 0.03	0.16 ± 0.02	0.3 ± 0.1
49	tr.	n.d.	tr.	0.03 ± 0.01
50	0.3 ± 0.1	0.12 ± 0.07	0.38 ± 0.10	0.6 ± 0.2
51	n.d.	n.d.	n.d.	tr.
52	tr.	0.06 ± 0.05	0.11 ± 0.05	0.3 ± 0.1
total alkyl esters	*0.5 ± 0.2*	*0.3 ± 0.2*	*0.8 ± 0.1*	*1.3 ± 0.6*

than their odd chain length homologs. Small amounts of odd chain length homologs were detected in both alcohols and aldehydes. Alkyl esters were dominated by the C_{50} chain length, made up of primarily C_{34} alcohol combined with C_{16} fatty acid.

Within the triterpenoid compound class, compounds identified included glutinol and its derivatives glutanol and glutinol acetate, friedelin and its derivative epifriedelanol, β-amyrin, and germanicol. Glutinol was the most abundant triterpenoid in both the epicuticular and intracuticular wax layers (fig. 3.4), making up 13 ± 4% of the epicuticular and 19 ± 5% of the intracuticular wax. Friedelin, the next most abundant triterpenoid, contributed 9 ± 1% and 8 ± 2% to the epicuticular and intracuticular wax layers respectively, while its derivative epifriedelanol made up 2-3% of the wax in each layer. Glutanol and germanicol were equally distributed in terms of µg/cm^2 through the epicuticular and intracuticular wax (fig. 3.4), but in terms of relative composition, both had a higher abundance in the intracuticular wax. Glutanol made up 2.0 ± 0.2% of the epicuticular and 5 ± 1% of the intracuticular wax, while germanicol contributed 2.6 ± 0.7% to the epicuticular wax and 7 ± 1% to the intracuticular wax.

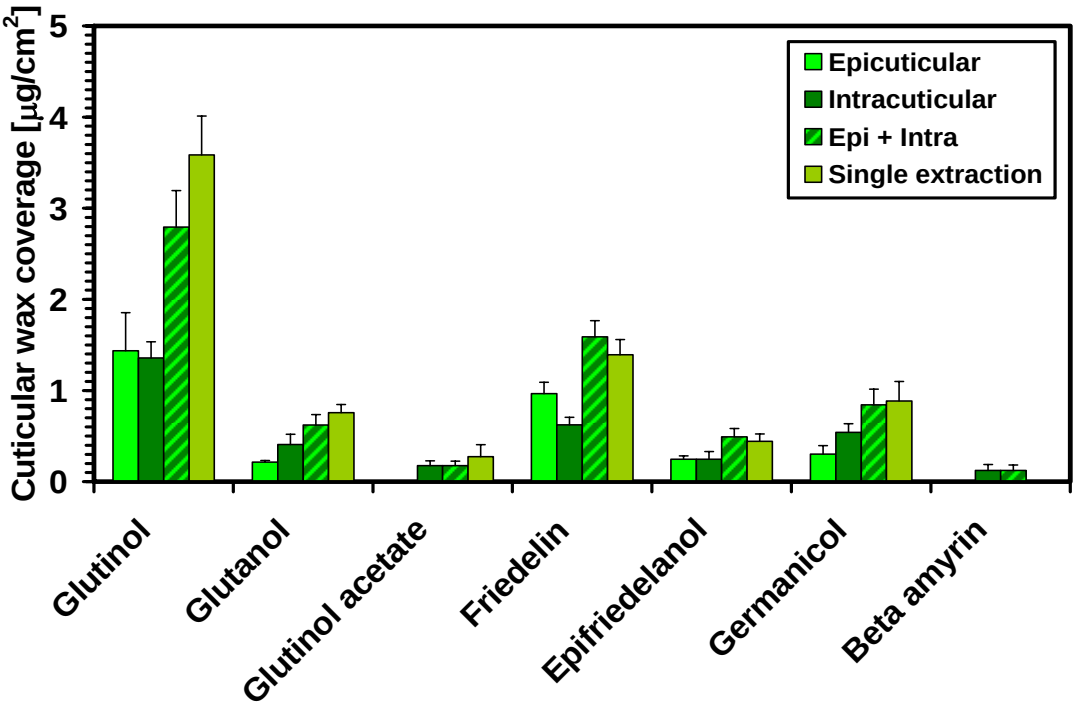

Figure 3.4: Composition of triterpenoid fraction of abaxial wax: n=5, error bars show standard error. Epi+intra = combined, single extraction = one step extraction of abaxial wax. Beta amyrin was not detected in single extraction due to co-elution with tritriacontane.

3.3.3: Adaxial wax composition

In analysis of the adaxial wax, the GA treatment yield (13.3 ± 0.7 µg/cm^2) was significantly higher than the $CHCl_3$ extraction yield (6.8 ± 0.7 µg/cm^2) (fig. 3.5)

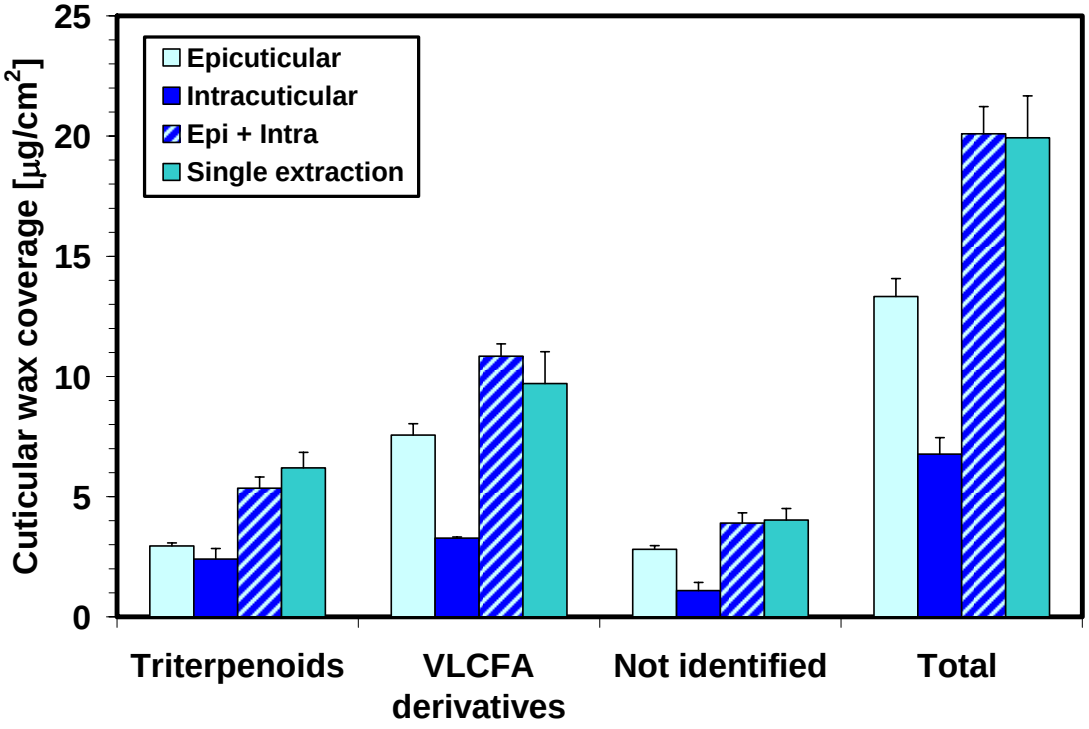

Figure 3.5: Adaxial wax composition by compound class: experiments were performed on four (epi, intra, combined (epi+intra)) or five (single extraction) parallels. Error bars show standard error.

Unlike the abaxial leaf surface, there was a detectable difference between the amount of aldehydes, fatty acids, and alkyl esters obtained in the two treatments, with all three more abundant in the epicuticular wax (fig. 3.6).

VLCFA derivatives were the most abundant group of compounds in the adaxial wax, making up 57 ± 1% of the epicuticular and 50 ± 6% of the intracuticular waxes. Triterpenoids contributed 22.2 ± 0.4% and 35 ± 4% to the epicuticular and intracuticular totals respectively. 21 ± 1% of the epicuticular and 16 ± 4% of the intracuticular wax remained unidentified.

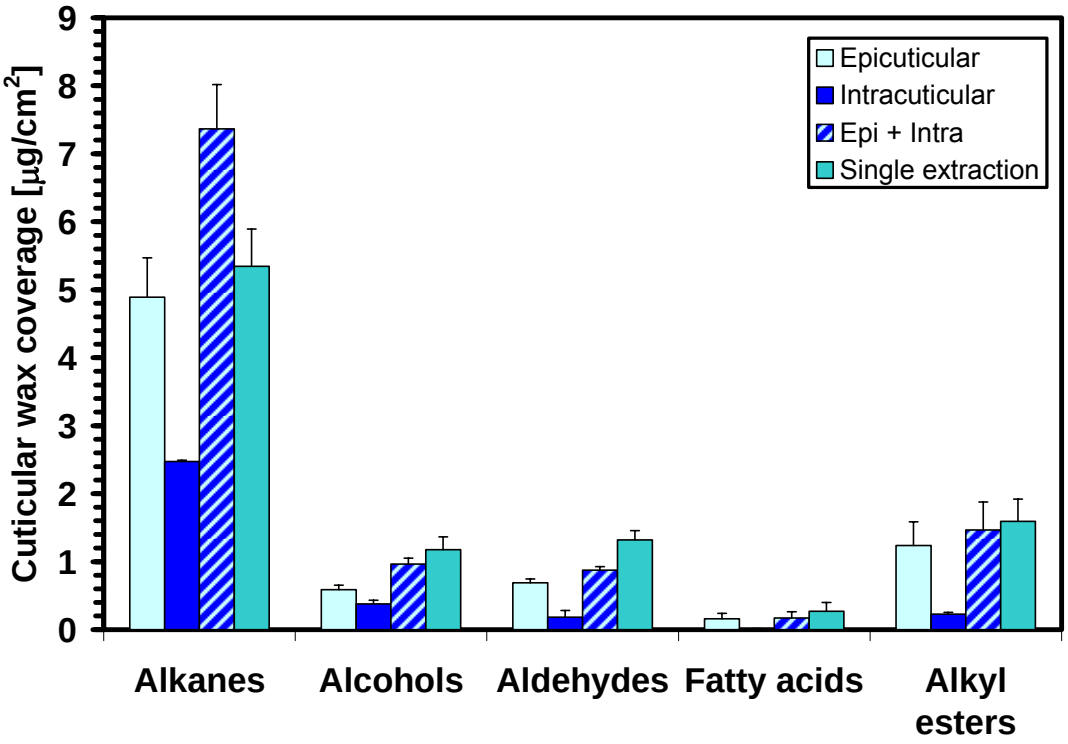

Figure 3.6: Composition of VLCFA derivative classes of adaxial wax: n = 4-5, standard error shown. Epi+intra = combined, single extraction = one step extraction

As in the abaxial wax, alkanes were the most abundant compound class among VLCFA derivatives (fig. 3.6) making up 36 ± 3% of the epicuticular and 37 ± 4% of the intracuticular waxes. The relative composition for most VLCFA derivatives was roughly equal in both wax layers with the exception of alkyl esters which made up 9 ± 3% of the epicuticular but only 3.4 ± 0.5% of the intracuticular wax. Chain length distributions for all compounds were the same as the abaxial surface (table 3.2).

Table 3.2: VLCFA chain length distribution of adaxial wax: Analyses were carried out on four (epi, intra, combined) or five (single extraction) parallels. Data are shown as mean ± standard error in µg/cm^2 for epicuticular, intracuticular, combined (epi+intra) and single extraction. tr. = trace, n.d. = not detected

	Epicuticular	Intracuticular	Epi + Intra	Single extraction
Alkane chain length				
29	tr.	0.14 ± 0.07	0.14 ± 0.07	0.032 ± 0.004
30	n.d.	tr.	tr.	n.d.
31	0.17 ± 0.03	0.5 ± 0.3	0.7 ± 0.3	0.28 ± 0.04
33	4.3 ± 0.5	1.6 ± 0.3	5.9 ± 0.8	4.5 ± 0.5
34	0.08 ± 0.04	n.d.	0.08 ± 0.04	0.13 ± 0.02
35	0.38 ± 0.06	0.08 ± 0.04	0.47 ± 0.08	0.38 ± 0.04
total alkanes	*4.9 ± 0.6*	*2.47 ± 0.02*	*7.4 ± 0.7*	*5.3 ± 0.5*
Alcohol chain length				
26	tr.	tr.	tr.	0.013 ± 0.004
28	0.10 ± 0.04	n.d.	0.10 ± 0.04	0.12 ± 0.01
31	0.015 ± 0.007	0.054 ± 0.009	0.07 ± 0.02	0.11 ± 0.02
32	0.16 ± 0.02	0.14 ± 0.05	0.30 ± 0.03	0.21 ± 0.03
33	tr.	0.03 ± 0.01	0.058 ± 0.005	0.13 ± 0.02
34	0.26 ± 0.04	0.12 ± 0.02	0.38 ± 0.04	0.5 ± 0.1
35	tr.	n.d.	tr.	0.05 ± 0.02
36	tr.	n.d.	tr.	0.06 ± 0.02
total alcohols	*0.588 ± 0.065*	*0.38 ± 0.05*	*0.97 ± 0.09*	*1.2 ± 0.2*
Aldehyde chain length				
32	0.28 ± 0.09	tr.	0.30 ± 0.09	0.37 ± 0.08
33	0.12 ± 0.01	n.d.	0.12 ± 0.01	0.12 ± 0.02
34	0.3 ± 0.1	0.2 ± 0.1	0.5 ± 0.1	0.8 ± 0.2
total aldehydes	*0.7 ± 0.1*	*0.2 ± 0.1*	*0.87 ± 0.05*	*1.3 ± 0.1*
Fatty acid chain length				
32	0.12 ± 0.07	tr.	0.13 ± 0.08	0.09 ± 0.06
34	0.039 ± 0.007	tr.	0.04 ± 0.01	0.18 ± 0.07
total fatty acids	0.16 ± 0.08	0.011 ± 0.005	0.17 ± 0.09	0.3 ± 0.1
Alkyl ester chain length				
42	tr.	n.d.	tr.	n.d.
44	tr.	n.d.	tr.	0.041 ± 0.005
46	0.06 ± 0.02	0.016 ± 0.009	0.07 ± 0.02	0.11 ± 0.01
47	n.d.	n.d.	n.d.	tr.
48	0.23 ± 0.07	0.08 ± 0.01	0.31 ± 0.09	0.31 ± 0.07
49	tr.	n.d.	tr.	0.04 ± 0.01
50	0.6 ± 0.2	0.136 ± 0.009	0.7 ± 0.2	0.7 ± 0.1
51	tr.	n.d.	tr.	0.04 ± 0.02
52	0.3 ± 0.1	n.d.	0.3 ± 0.1	0.37 ± 0.08
total alkyl esters	*1.2 ± 0.3*	*0.23 ± 0.03*	*1.5 ± 0.4*	*1.6 ± 0.3*

Triterpenoid composition (in µg/cm^2) for the adaxial leaf surface was, for the most part, comparable with the abaxial wax (fig. 3.7). One notable difference was the higher abundance of friedelin in the epicuticular wax layer in the adaxial wax which was not observed in analysis of the abaxial wax. This difference was reflected in the relative composition, with friedelin making up 6.7 ± 0.3% of the epicuticular wax and only 3.9 ± 0.2% of the intracuticular wax. In terms of relative composition, both glutinol and glutanol were more abundant in the intracuticular wax, where they made up 14 ± 2% and 6.3 ± 0.6% respectively, than the epicuticular, where glutinol contributed 7.9 ± 0.3% and glutanol made up only 2.9 ± 0.4%.

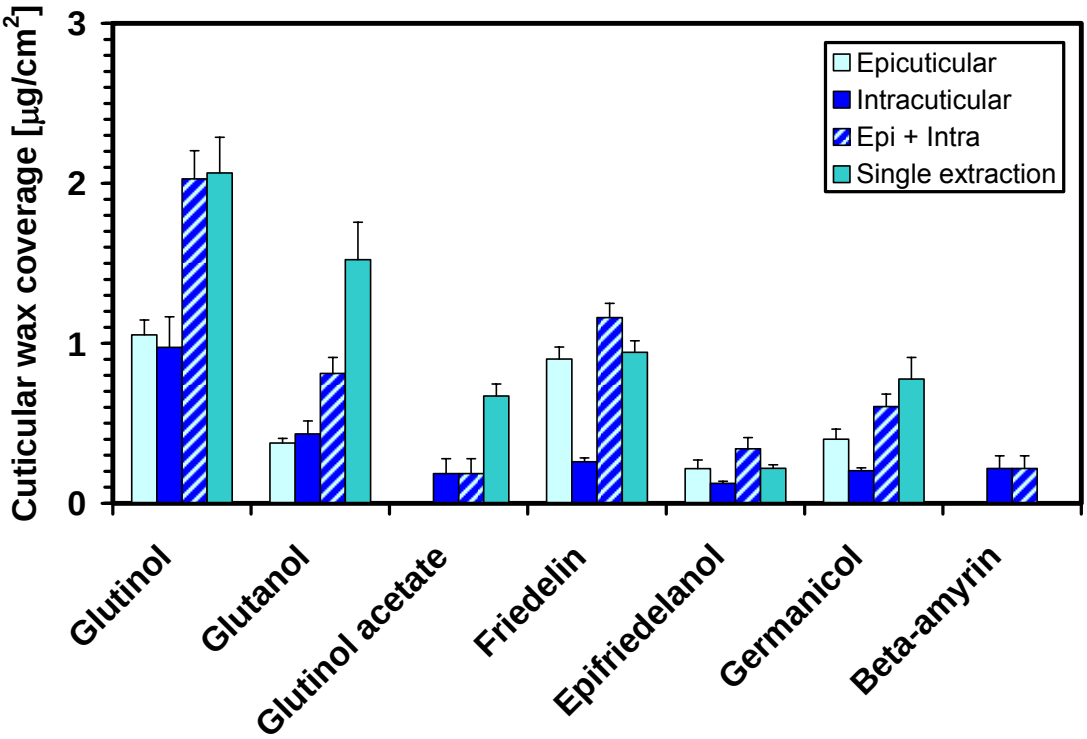

Figure 3.7: Composition of triterpenoid fraction of adaxial wax: n = 4-5, standard error shown. Epi + intra = combined, single extraction = one step wax removal. Beta-amyrin was not detected in single extraction due to co-elution with tritriacontane.

3.3.4: Total wax composition

In order to compare the yield of GA treatment + $CHCl_3$ extraction with a meaningful total, a single wax extraction (using $CHCl_3$) was performed for each leaf surface. The total wax coverage for each surface was approximately 20µg/cm^2.

Cuticular wax in *K. daigremontiana* was dominated by VLCFA derivatives (fig.3.2, 3.5) which made up 45-50% of the total on both the abaxial and adaxial leaf surfaces. 25-35% of the wax was composed of triterpenoids, while unidentified compounds contributed 15-20% to the total.

The only compounds for which statistically significant differences in coverage were seen between the adaxial and abaxial surfaces were glutinol and glutanol. Glutanol was more abundant in the adaxial wax (1.5 ± 0.2 μg/cm^2) than the abaxial (0.76 ± 0.09 μg/cm^2), while glutinol was found in higher abundance in the abaxial wax (3.6 ± 0.4 μg/cm^2 vs. 2.2 ± 0.2 μg/cm^2 in the adaxial cuticular wax).

3.3.5: Physical characterization of epicuticular wax on adaxial and abaxial surfaces

Crystals were seen on both the abaxial and adaxial leaf surfaces (fig. 3.8). Crystal distribution was not uniform when comparing neighbouring cells, and crystals appeared to be more plentiful on the abaxial surface than the adaxial surface. Crystals were clustered together rather than being evenly distributed. Some cells had denser crystal coverage than others (fig. 3.9(A)), and there was no visible difference between the crystal arrangement on guard cells compared to pavement cells (fig. 3.9(B)). Two crystal shapes were observed, which can be described as platelets with sinuate margins (fig. 3.8(C) – indicated by arrows) and twisted ribbons (fig. 3.8(F) – indicated by arrows).

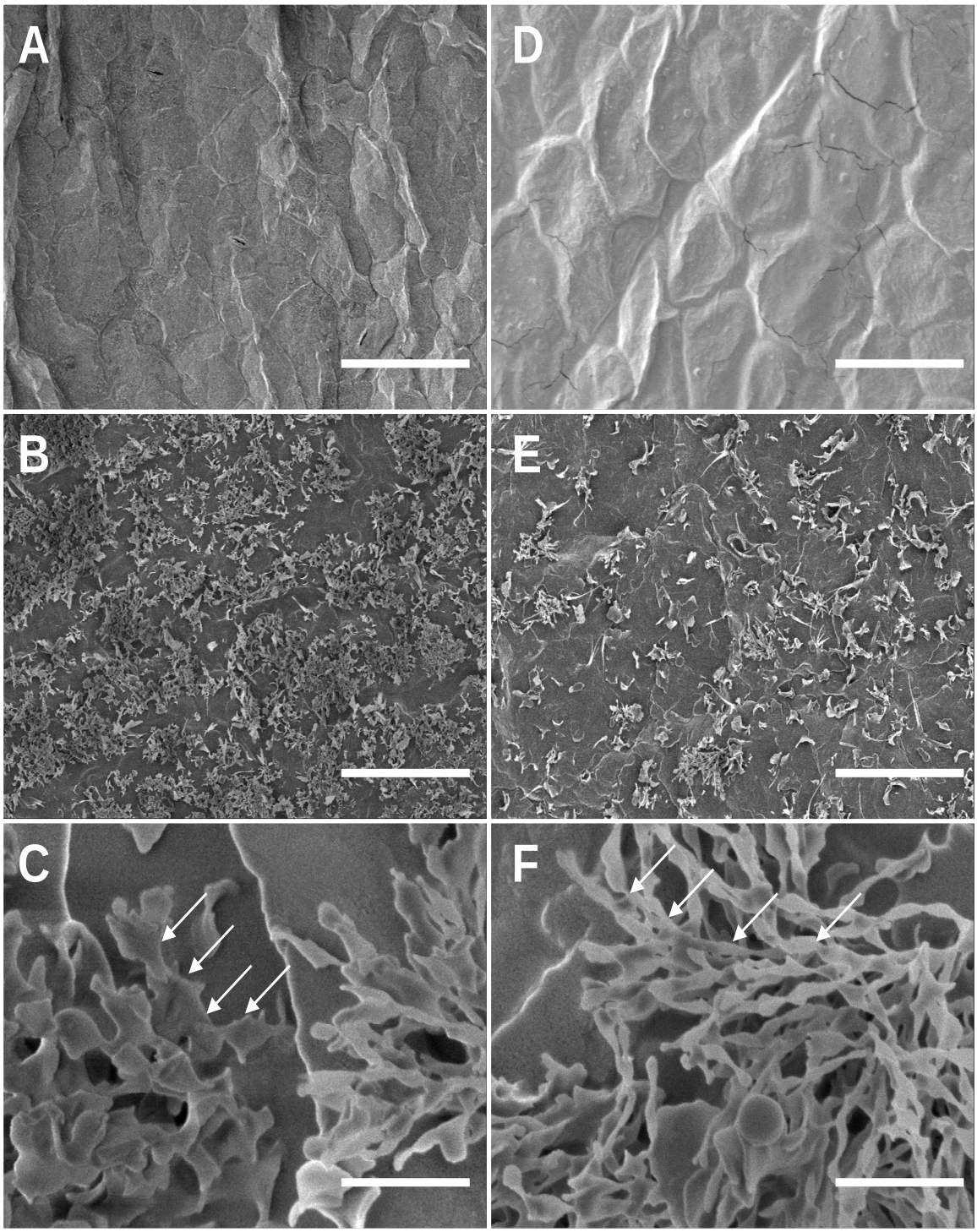

Figure 3.8: SEM images of abaxial (A-C) and adaxial (D-F) leaf surfaces: Images were taken at 200X (A,D), 2000X(B,E) and 20 000X(C,F) magnifications, with scale bars representing 100 µm, 10 µm, and 1 µm respectively. Both platelet like (C – indicated by arrow) and twisted ribbon (F – indicated by arrow) crystals were observed.

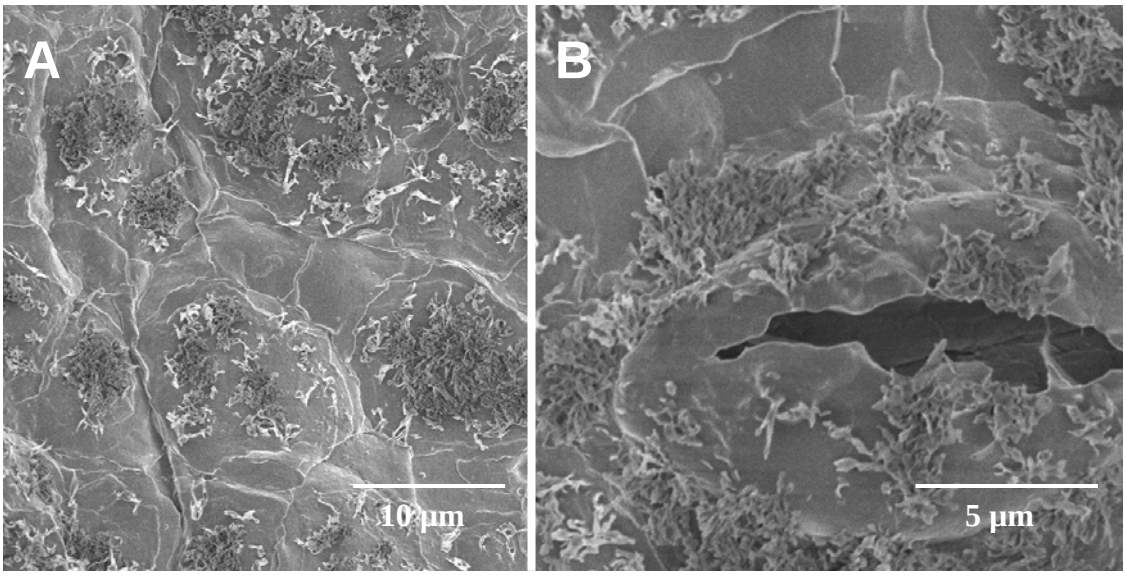

Figure 3.9: SEM images of abaxial leaf surface: A) showing uneven coverage of epicuticular wax crystals, B) crystal coverage on pavement and guard cells surrounding a stoma. A was taken at 2 000 X, B at 5 000 X.

3.4: Discussion:

Comparing adaxial and abaxial analyses reveals that, for the most part, there is very little difference in wax composition between the two surfaces. There are, however, a few differences of possible interest.

The abaxial leaf surface has a significantly higher concentration of glutinol than the adaxial surface in both absolute (see results) and relative terms (18 ± 2% vs. 11.2 ± 0.6%; mean ± standard error). Glutanol, on the other hand, is found in higher abundance on the adaxial surface (7.5 ± 0.6% vs. 3.8 ± 0.3%). The adaxial leaf surface is exposed to more direct sunlight, falling debris, and rain water than the underside of the leaf. Perhaps the distribution of glutinol and glutanol is an adaptation to one of these or another variable. Without further experiments, there is no way to know what (if any) possible advantage there is to the plant of having the glutinol and glutanol differentially distributed between the two leaf surfaces.

The fact that glutanol is the reduced form of glutinol and presumably derived from the former by a single reaction suggests the presence of an enzyme which converts

some of the glutinol to glutanol before it can be excreted to the surface of the plant. This enzyme might operate preferentially in the adaxial epidermis. The sum of glutinol and glutanol for the abaxial surface and adaxial surface are statistically equivalent (4.3 ± 0.5μg/cm^2 and 3.4 ± 0.4μg/cm^2 respectively, mean ± standard error, p=0.14, 2 tailed t-test) which makes it unlikely that there is a difference in OSC activity between the adaxial and abaxial leaf surfaces. More likely there is a difference only in the reductase activity.

Although the total wax coverage was equal for both sides of the leaf, there was a significant difference between epi and intracuticular wax amounts for the adaxial but not the abaxial wax, with the adaxial surface having more epicuticular than intracuticular wax (fig. 3.1, fig. 3.5). If the division of compounds into epicuticular and intracuticular layers is a matter of interactions between triterpenoids and VLCFA derivatives, then these effects should be the same on both sides of the leaf, resulting in similar or identical wax profiles.

Since the total extractable wax on each surface is the same, a thicker cutin/intracuticular wax layer on the abaxial surface than the adaxial could lead to abaxial epicuticular wax, which can be removed with gum arabic, being thinner. This may be confirmed or denied by analysis of isolated cuticles and cutin for the two surfaces.

There is a possibility that the difference between epicuticular and intracuticular wax layers seen on the adaxial surface is representative of both leaf surfaces and that the abaxial intracuticular analysis was contaminated by residual epicuticular wax. The abaxial surface has a more glaucous appearance and denser crystal coverage. Denser coverage of crystals has implications for the wettability of the surface and may mean that the gum arabic can not adhere to and remove the epicuticular wax as effectively as on a less densely covered surface. Because of this, a single gum arabic peel may not have been as complete as on the adaxial surface. This potentially non-exhaustive removal of abaxial epicuticular wax may have contributed to differences noted between the adaxial and abaxial surfaces in terms of epi/intra abundances for several compounds.

Working with this assumption, we can make some estimates about the degree to which the epi/intra division might be misrepresented. These estimates are meant to illustrate the extreme case based on an assumption of the epi/intra ratios being equal for

both leaf surfaces. It is not clear that this is true, but a possibility that is worth considering. Based on the successive gum arabic peels performed on the adaxial surface, up to ~4 $\mu g/cm^2$ of epicuticular wax can be left behind after the first gum arabic treatment and thus appear in the chloroform extract and be misinterpreted as intracuticular wax. For the abaxial surface, the yield from the first gum arabic treatment was lower than that of the first treatment of the adaxial surface (~10 $\mu g/cm^2$ vs. ~13 $\mu g/cm^2$). Together, these results mean that up to ~7$\mu g/cm^2$ of the chloroform extracted wax, interpreted as intracuticular wax in the abaxial analysis could actually be residual epicuticular wax. This affects the quality and quantity of intracuticular wax recovered (since epicuticular material could have been mistakenly included) as well as the quantity of epicuticular wax. The relative composition of the epicuticular wax should not be affected, because physical removal with gum arabic would not remove intracuticular wax, thus precluding the possibility of cross contamination. To determine if this potentially incomplete epicuticular wax removal is the cause of the discrepancy observed between the adaxial and abaxial wax analyses (which may or may not be representative of the actual cuticle composition), repeated experiments would be necessary. Additionally, repeated gum arabic treatments of the abaxial leaf surface could be performed, as on the adaxial surface, to assess the number of peels required for exhaustive removal of epicuticular wax. Repeated gum arabic treatments were not initially performed for the epicuticular/intracuticular analyses because of the fragility of the leaves and the fact that a single wax peel removed most of the wax in the parallel experiment.

If the abaxial gum arabic treatment left behind a significant amount of epicuticular wax, one might expect that the relative compositions of the two fractions (GA treatment or epicuticular and $CHCl_3$ extract or intracuticular) would be more similar than in the case of the potentially better separated adaxial samples. However, this does not appear to be the case. Triterpenoids made up 29 ± 5% of the epicuticular and 46 ± 5% of the intracuticular wax in the abaxial analysis, but 22.2 ± 0.4% and 35 ± 4% of the two wax layers respectively for the adaxial surface. VLCFA derivatives, on the other hand, make up 52 ± 4% of epicuticular and 34 ± 4% of intracuticular wax for the abaxial wax, but 57 ± 1% and 50 ± 6% of the two wax layers respectively for the adaxial surface. The fact that the epi/intra profiles are more similar for the adaxial wax contradicts

expectations and makes a definitive explanation of the observed results impossible without further investigation. The analysis could be further complicated by the fact that gradients between epicuticular and intracuticular wax layers may not be equal or even similar for the two leaf surfaces. Repeated epicuticular wax peels might be worth doing to ensure exhaustive removal of abaxial epicuticular wax.

For both the adaxial and abaxial leaf surfaces, there was a gradient in triterpenoid concentration, which made up relatively more of the intracuticular wax layer for each surface, and VLCFA derivatives, which made up a higher proportion of the epicuticular wax. Although neither compound class was found exclusively within one wax layer, the gradients observed appear to agree in principle with those reported for several species with cuticular triterpenoids.[13-15]

In the adaxial wax, there seems to be a greater difference between the epicuticular and intracuticular wax coverages (in $\mu g/cm^2$) with respect to most VLCFA derivatives (fig. 3.3, 3.6). In the adaxial wax these compounds are found primarily in the epicuticular layer, while in the abaxial wax they are evenly distributed between the two layers. However, in this case the relative composition tells a different story. Alkanes make up a larger proportion of the epicuticular than intracuticular wax for the abaxial surface, but 36%-38% of each layer for adaxial wax. Thus the abaxial but not the adaxial wax shows a gradient in alkane concentration. In most other compound classes, the difference between their relative composition in the epi and intracuticular layers is neither large nor significant. Alkyl esters, however, were relatively more abundant in the epicuticular wax than the intracuticular layer in the adaxial wax only.

Friedelin marks a departure from the general pattern of triterpenoids being found in higher abundance in the intracuticular wax. In the adaxial wax friedelin is found preferentially in the epicuticular wax, but less of a gradient is evident in the abaxial wax. If friedelin is indeed found mainly in the epicuticular wax, it may be due to its ketone functionality, which is unique among the triterpenoids found in *K. daigremontiana*. Germanicol, glutinol, and glutanol, which have hydroxyl functional groups, are all found in relatively higher concentrations in the intracuticular wax. β-amyrin was found exclusively in the intracuticular wax layer, but this should not be over-interpreted as its retention time is very similar to tritriacontane in the GC-FID and GC-MS conditions

used. In the epicuticular and total wax extractions, the smaller peak is completely obscured by the large alkane peak, making identification and quantification impossible.

Based on qualitative analysis of SEM images, it appears that the twisted ribbon type crystals (fig. 3.8(F)) are more abundant on the adaxial leaf surface, although they can be seen on both surfaces. The irregular platelets appear on both surfaces. Crystals appear to be more plentiful on the abaxial surface than the adaxial (fig. 3.8), yet epicuticular wax coverage per unit area is greater on the adaxial surface. It would appear that either the images of crystals are not representative of the entire leaf's wax coverage, or the epicuticular wax film accounts for a significant portion of the epicuticular wax fraction. Another alternative, suggested earlier, may be that single gum arabic peels of the abaxial surface were not exhaustive in removal of epicuticular wax.

Having a chemical picture of the wax composition as well as an idea of what the surface looks like under a microscope, we can attempt to relate the chemical and physical data. It is generally accepted that wax crystals form when there is a high concentration of a compound within the epicuticular wax. The major component of the epicuticular wax is usually considered to be the major component of the wax crystals, but in this case, the largest fraction (although less than 40%) of the epicuticular wax is comprised of alkanes, but the crystals do not appear to resemble alkane crystals found in other species. Alkane crystals have been reported to appear as rodlets, platelets, dendritic rodlets, crust, and transversally ridged rodlets [9, 19] none of which accurately describe the crystals on the leaves of *K. daigremontiana*. On the surface of pea leaves, crystals on the abaxial surface containing mostly C_{31} alkane took the shape of upright ribbons.[18] These ribbon crystals are more similar in appearance to those observed on the leaves of *K. daigremontiana* than the other alkane containing crystals, and the most abundant single compound in the epicuticular wax of *K. daigremontiana* is tritriacontane (C_{33} alkane) which has a very similar structure to its C_{31} homolog. However, the ribbons seen in *K. daigremontiana* are twisted. It is possible that crystals on *K. daigremontiana* are made up of a mixture of compounds which co-crystalize. Analysis of epicuticular wax on pea leaves found dramatically different crystals on the adaxial and abaxial leaf surfaces containing the same compounds, but in very different ratios.[18] The unique combination of alkanes,

alcohols, and other VLCFA derivatives in *K. daigremontiana* wax could contribute to the formation of the twisted ribbon shaped crystals.

Another possibility, raised by the fact that triterpenoids in this wax are present in both the epicuticular and intracuticular waxes, could be that the crystals observed on the surface contain triterpenoids. Crystals of many different shapes have been reported to contain triterpenoids. Some of the shapes reported include platelets, polygonal rodlets, threads, and plates.[8-10] While none of these shapes accurately describe the crystals seen on *K. daigremontiana* (with the possible exception of a variation on platelets) it is not impossible that crystals of glutinol, glutanol, friedelin, epifriedelanol or some combination of these compounds might look different to those triterpenoid crystals which are already known.

The presence of two different types of epicuticular wax crystals in *K. daigremontiana* means that each type of crystal could result from a different chemical composition. Maybe the twisted ribbons contain alkanes and other VLCFA derivatives while the irregular platelets contain one or more of the triterpenoids. Due to the difference in shape between the two classes of molecules, it is unlikely that the crystals contain both VLCFA derivatives and triterpenoids. If both classes of compounds were present in the crystals, one might expect to see a range of crystal shapes between the two extremes of ribbons and platelets, made up of varying triterpenoid/VLCFA ratios. Since this is not observed, it seems likely that each type of crystal has a characteristic composition. Confirmation of this would require either a way of physically isolating and separating the crystals and then collecting enough of each for chemical analysis (which would be nearly impossible) or some way of analyzing their composition in situ. Recent work in our department has opened up the possibility of using techniques such as Raman micro-spectroscopy to localize triterpenoids on the cellular level.[20] It may not be possible yet to achieve fine enough resolution to identify crystals using this method, but continued development of this and other methods may allow us to take a "chemical picture" of the crystals and definitively establish their chemical composition.

If the two crystal forms have different compositions, there are implications for crystal formation. It is possible that the triterpenoid containing crystals form early on in leaf development when the relative concentration of triterpenoids in the wax is high. This

would effectively remove some or all of the triterpenoids from the bulk of the epicuticular wax, increasing the relative concentration of VLCFA derivatives available for formation of the second crystal type, especially as the relative amount of VLCFA derivatives increases during leaf ontogenesis. Establishing definitively the mechanism through which these crystals form would require a thorough analysis of leaves in different stages of development. Information about the epicuticular and intracuticular wax composition would be needed for leaves in different stages of development, as would SEM images to allow comparison of crystal structure and composition between different leaves.[21]

3.5: Conclusion:

Analysis of the epicuticular and intracuticular waxes on both the adaxial and abaxial surfaces of *K. daigremontiana* leaves was performed. Both adaxial and abaxial waxes were dominated by VLCFA derivatives, which were in turn dominated by alkanes. Alkanes and some other VLCFA derivatives were found mainly in the epicuticular wax, although they were present in the intracuticular waxes as well. In the triterpenoid fraction, glutinol was the most abundant compound and was distributed equally between the epicuticular and intracuticular wax layers. Glutinol and its derivative glutanol were the only compounds for which a difference in coverage was detected between the adaxial and abaxial surfaces. It is hypothesized that this is due to a difference in the activity of an enzyme converting glutinol to glutanol rather than a difference in OSC activity between the two leaf surfaces. Epicuticular wax crystals were found on both sides of the leaf with two different shapes that could be described as twisted ribbons and irregular platelets. The chemical composition of these crystals could not be determined with the information currently available, but it is possible that the two types of crystals could be made up of VLCFA derivatives (twisted ribbons) and triterpenoids (irregular platelets).

References

(1) Krauss, P.; Markstadter, C.; Riederer, M. *Plant Cell Env.* **1997**, *20*, 1079-1085.

(2) Barthlott, W.; Neinhuis, C. *Planta* **1997**, *202*, 1-8.

(3) Eigenbrode, S. D.; Espelie, K. E. *Annu. Rev. Entomol.* **1995**, *40*, 171-194.

(4) Eigenbrode, S. D.; Pillai, S. K. *J. Chem. Ecol.* **1998**, *24*, 1611-1627.

(5) Eigenbrode, S. D.; Jetter, R. *Integ. Comp. Biol.* **2002**, *42*, 1091-1099.

(6) Gorb, E.; Haas, K.; Henrich, A.; Enders, S.; Barbakadze, N.; Gorb, S. *J. Exp. Biol.* **2005**, *208*, 4651-4662.

(7) Riedel, M.; Eichner, A.; Jetter, R. *Planta* **2003**, *218*, 87-97.

(8) Markstadter, C.; Federle, W.; Jetter, R.; Riederer, M.; Holldobler, B. *Chemoecology* **2000**, *10*, 33-40.

(9) Barthlott, W.; Neinhuis, C.; Cutler, D.; Ditsch, F.; Meusel, I.; Theisen, I.; Wilhelmi, H. *Bot. J. Linn. Soc.* **1998**, *126*, 237-260.

(10) Jeffree, C. E. In *Fine structure of the plant cuticle;* Riederer, M., Muller, C., Eds.; Biology of the Plant Cuticle; Blackwell Publishing: UK, 2006; Vol. 23, pp 11-110.

(11) Jetter, R.; Riederer, M. *Planta* **1994**, *195*, 257-270.

(12) Meusel, I.; Neinhuis, C.; Markstadter, C.; Barthlott, W. *Can. J. Botany* **1999**, *77*, 706-720.

(13) Jetter, R.; Schaffer, S.; Riederer, M. *Plant Cell Env.* **2000**, *23*, 619-628.

(14) Buschhaus, C.; Herz, H.; Jetter, R. *Ann. Botany* **2007**, *100*, 1557-1564.

(15) Buschhaus, C.; Herz, H.; Jetter, R. *New Phytol.* **2007**, *176*, 311-316.

(16) Peschel, S.; Franke, R.; Schreiber, L.; Knoche, M. *Phytochemistry* **2007**, *68*, 1017-1025.

(17) Leide, J.; Hildebrandt, U.; Reussing, K.; Riederer, M.; Vogg, G. *Plant Physiol.* **2007**, *144*, 1667-1679.

(18) Gniwotta, F.; Vogg, G.; Gartmann, V.; Carver, T. L. W.; Riederer, M.; Jetter, R. *Plant Physiol.* **2005**, *139*, 519-530.

(19) Ensikat, H. J.; Boese, A.; Mader, W.; Barthlott, W.; Koch, K. *Chem. Phys. Lipids* **2006**, *144*, 45-59.

(20) Yu, M. M. L.; Konorov, S. O.; Schulze, H. G.; Blades, M. W.; Turner, R. F. B.; Jetter, R. *Planta* **2008**, *227*, 823-834.

(21) Yu, M. M. L.; Schulze, H. G.; Jetter, R.; Blades, M. W.; Turner, R. F. B. *Appl. Spectrosc.* **2007**, *61*, 32-37.

Chapter 4: Conclusions and future work

This research, while focused on *Kalanchoe daigremontiana*, dealt with the larger questions of wax and triterpenoid biosynthesis. We are interested in the characterization of a glutinol and/or friedelin synthase as well as establishing the role of triterpenoids within the cuticle. Finding out more about how different compounds are sorted into epicuticular and intracuticular wax layers as well as how epicuticular crystals form is another area of interest. What follows is a summary of some of the links observed between the different parts of the project, possible insights into these and other questions, and potential future experiments.

From a systematic time course analysis of wax composition in *K. daigremontiana*, it was concluded that the wax composition of leaves at the third node changed from being primarily (~70%) triterpenoids in young leaves to about 50% triterpenoids in 140 day old leaves. Analysis of adaxial and abaxial waxes of larger mature leaves for the second study (as reported in chapter 3) determined that triterpenoids made up only 25-35% of cuticular wax for these leaves. Correspondingly, the proportion of VLCFA derivatives in the mature leaves analyzed was higher (45-50%) than in the 140 day old leaves from the time course (~35%). While it appears from the time course experiment that wax biosynthesis has stopped by 140 days for the third leaf pair, it is possible that had the leaves remained on the plant longer, further changes in wax composition may have been observed. It is also possible that the larger upper leaves, which grow and apparently mature more quickly (fig. 2.1), also undergo a more dramatic change in wax composition, thus changing from being dominated by triterpenoids to being dominated by alkanes and other VLCFA derivatives. Several other analyses of the wax composition in mature leaves (data not shown) support the observed cuticular wax composition as noted in the mature leaves analyzed for chapter 3. Within both the time course experiment and the subsequent epicuticular/intracuticular analyses, relative abundances within compound classes were consistent. Tritriacontane was by far the most abundant of the VLCFA derivatives in all leaves analyzed, while glutinol remained the most abundant triterpenoid even when the relative proportion of triterpenoids decreased.

Early in leaf development, when triterpenoid content within the wax was relatively high, epicuticular and intracuticular wax analyses were not completed. However, working with what is known from the mature leaf wax composition, it is

possible to make a few estimates. Mature leaves contain approximately 25-35% triterpenoids in their bulk wax and 22-29% triterpenoids in their epicuticular wax, thus triterpenoids are under-represented in the epicuticular wax. Extending this to the wax of young leaves, which contain ~ 70% triterpenoids, one could estimate that 60% of the epicuticular wax might be composed of triterpenoids. This would have implications for crystal formation, as discussed in chapter 3, and could allow for the formation of epicuticular triterpenoid crystals in young leaves followed by an accumulation of VLCFA derivatives and the subsequent formation of a second type of crystals.

Given time and resources, several more experiments could be undertaken to further investigate questions which have been raised by this investigation. Suggestions follow for some of these experiments.

During the time course experiment, a whole flat of plants was stripped of leaves for extraction and NMR analysis. The stumps of the plants were left unattended and not watered for several weeks. Upon examination, new growth was evident, but the new leaves and stems were a slightly different shape and a slightly lighter colour than the other leaves on the plant (fig. 4.1). It would be interesting to do a time course study of cuticular wax composition of the secondary growth arising after plants have been denuded of leaves. Comparison of this information with the existing time course information might help answer questions about how production of various components is controlled as well as maybe giving clues as to the function of the various compounds found within the cuticle.

Combining the two aspects of this investigation (time course and epicuticular wax characterization) leads to the possibility of yet another experiment. Answering the question of how crystals form on the leaves of *K. daigremontiana* would require a thorough time course analysis of epicuticular wax composition as well as SEM analysis for each wax analysis time point to enable comparisons to be made.

Behind the *K. daigremontiana* project are larger questions about the function of triterpenoids in cuticular wax and their biosynthesis. The role of triterpenoids in the cuticle is as yet unknown, but they may play a role in moderating cuticle permeance or protecting the plant during interactions with potential pests.

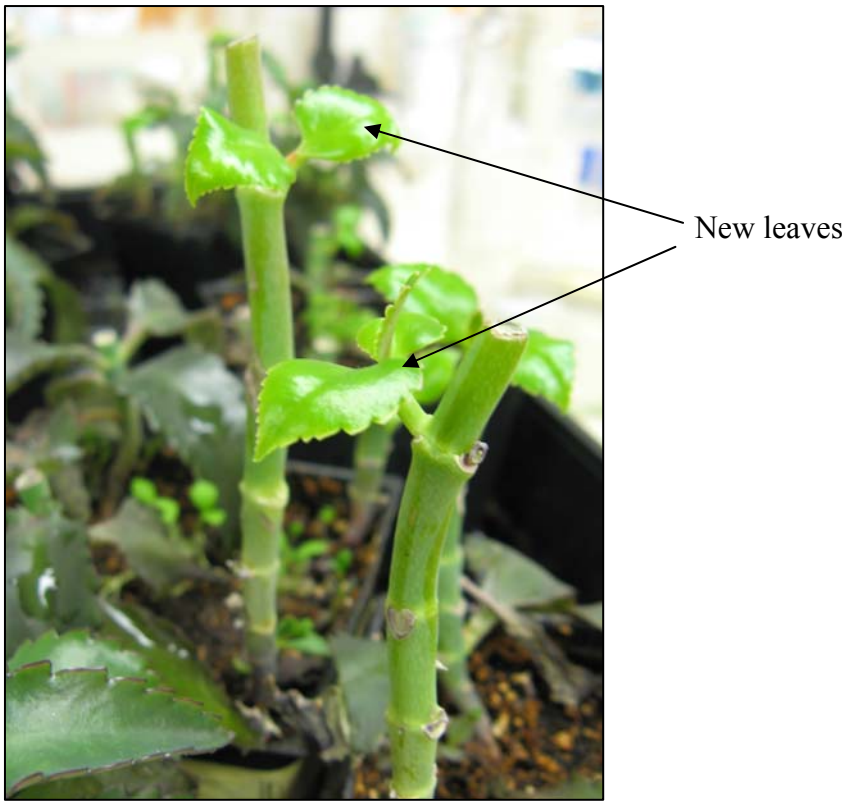

Figure 4.1: New growth on *K. daigremontiana* stumps: leaves were removed for analysis and stumps left without water for approximately two weeks

The question of whether triterpenoids affect cuticular permeance could be approached through heterologous expression of a triterpenoid synthase gene in an appropriate host plant. Another avenue of investigation to address the same question could be analysis of the permeance of cuticles from *K. daigremontiana* leaves of different ages. Since the relative changes in triterpenoid and VLCFA derivative concentrations during leaf development are known, any corresponding change in the water barrier effectiveness could contribute to our understanding of the role of triterpenoids. However, this approach does not take into account other changes which may be occurring in the cuticle, such as changes in cutin or other components which are not monitored.

Since triterpenoids are found in the epicuticular wax of *K. daigremontiana*, they are available for interaction with insects or other potential pests and may play a protective role for the plant. Through an analysis of any ecological literature relating to this species, one might find a pathogen, insect, or infection that targets *K. daigremontiana*. The

resistance of leaves of various ages to the agent could be tested, and activity guided fractionation might then allow determination of any active component within the cuticular wax.

Heterologous expression of triterpenoid synthase genes would provide another route for further investigation. Once the functions of these genes have been confirmed, they could be expressed in yeast. This process could easily be scaled up to obtain more of the triterpenoid product of interest for tests of biological or ecological activity. This would again require a suitable pathogen or insect of interest and deterrent effects that were measurable. Alternatively, genes from *K. daigremontiana* could be expressed in another plant system which is normally triterpenoid poor and may provide a better model of a pathogen/host system.

In addition to questions about a possible role of triterpenoids, there are questions about both activity and localization of the genes and enzymes involved in the process. Creation of constructs with appropriate markers (GFP) could allow protein localization on both a plant tissue level and on the cellular scale, while RT-PCR of candidate tissues in different stages of development would give a picture of when genes are actively being transcribed. Transformation for over expression in *K. daigremontiana* would not be a trivial process, but transformation into tobacco or Arabidopsis could give valuable information, in addition to a background relatively low in triterpenoids.

Along the avenue of triterpenoid biosynthesis, characterization of candidate genes based on sequence homology continues in our lab. The eventual goal of the project is to learn more about how the OSCs work and which amino acids are important in determining product specificity. Targeting key amino acids by site directed mutagenesis to try and change the product profile might be one way of determining the role of specific amino acids within the enzyme. Once a friedelin and/or glutinol synthase is positively identified, work to compare its structure with other OSC structures can begin, as well as efforts to modify its product profile by site directed mutagenesis.

The first stage of pentacyclic triterpenoid cyclization, involving successive ring closures by π-electron attack, is common to all pentacyclic triterpenoid structures. Thus the migration of the positive charge from the protonated epoxide to the other end of the molecule (in the E ring) must also be stabilized by all enzymes producing these

molecules. On the other hand, migration of the positive charge by methyl and hydride shifts proceeds to a different extent depending upon the molecule and the site of final deprotonation. Since the formation of friedelin or glutinol requires extensive charge migration, the enzyme involved must be able to accommodate and stabilize the positive charge as it moves from the E ring back to the A ring before deprotonation. Appropriate mutation(s) have the capacity to alter the product profile by terminating charge migration earlier, resulting in premature deprotonation products. Understanding which amino acids are involved in stabilizing the migrating positive charge and how changing these residues affects the product profile can add to an understanding of the triterpenoid synthesis mechanism.

Although this investigation does not provide answers to all of the questions posed at the outset, it provides some information which can contribute towards a fuller understanding of wax and triterpenoid biosynthesis. This is the first time, as far as we are aware, that a comprehensive time course analysis as well as a comprehensive spatial analysis of cuticular wax in *K. daigremontiana* has been reported. It is hoped that further investigations will make use of what is reported here in order to advance our knowledge of this complex field.

Appendix I: Cloning of *Kalanchoe* triterpenoid synthase (KTS) genes

A.1: Summary of cloning procedures

Full length cDNA sequences of several putative triterpenoid synthases from *K. daigremontiana* were prepared by a previous student. All of the sequences shared high homology (>70% nucleotide and >76% amino acid identity not including KCS genes which were putatively assigned as cycloartenol synthases), were approximately 2.3kb in length, and coded for proteins of ~760 amino acids. Three of the KTS genes had been characterized in preliminary experiments, so I chose five other genes to work on (fig. A1). My goal was to express these genes in yeast and determine the function of each. What follows is a summary of the steps taken with one sequence (KTS 13-9), which differed from what was attempted with the other sequences only very slightly (some primers were different; some techniques were attempted more or less frequently on various sequences). Ultimately, the results obtained were very similar for all sequences.

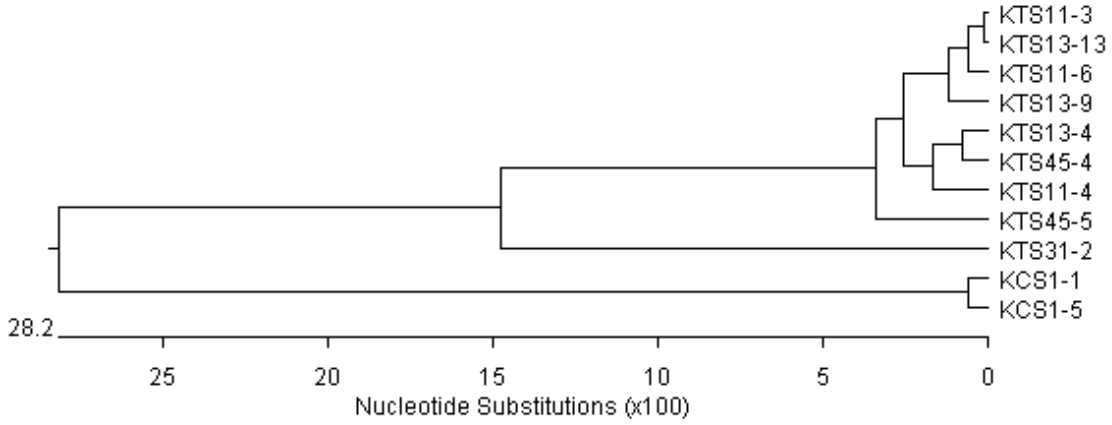

Figure A1: Phylogenetic tree of KTS amino acid sequences: Tree was prepared using Megalign program (DNA star). KTS 13-13, 45-5, and 45-4 had been partially characterized. I worked on characterization of KTS 11-3, 11-6, 13-9, 11-4, and 13-4. The steps described in the text are for KTS 13-9. Sequences for KCS1-1 and KCS1-5 suggest that these code for cycloartenol synthases

Based on sequence information, primers were designed (KTSC1S – forward primer, KTSC1A – reverse primer) to introduce two different restriction sites into the sequence, one at the beginning (HindIII) and one at the end (NotI). These restriction sites were chosen to correspond to those available in the yeast transfer vector pYES2

(Invitrogen) as well as being sites that were not found within the sequence of interest. Primer sequences are given in table A1.

Table A1: Primers used in molecular biology work: restriction sites are denoted by bold font and are identified in the third column.

Primer Name	Primer Sequence	Restriction site (bold)
T7	TAATACGACTCACTATAGGG	N/A
KTSC1S	GC**AAGCTT**AGGATGTGGAAGTTAAAGATAG	HindIII
KTSC1A	GC**GCGGCCGC**CTATTTAGACGGTTTTG	NotI

Using the primers KTSC1S and KTSC1A, PCR-amplification was performed with Phusion high fidelity polymerase (New England Biolabs) using 2µL of template, 1µL of each primer, 4µL 5X Phusion buffer (NEB), 0.4µL dNTP, 0.2µL Phusion high fidelity polymerase (NEB), 11.4µL H_2O (deionized and autoclaved) with the program: 98°C- 30s, 5 x (98°C-10s, 50°C-30s, 72°C-45s), 5 x (98°C-10s, 58°C-30s, 72°C-45s), 25 x (98°C-10s, 66°C-30s, 72°C-45s), 72°C-10min. This reaction was performed on a Mastercycler Gradient thermocycler (Eppendorf, Germany) as were all PCR-amplification reactions in the investigation. Subsequent analysis by gel electrophoresis (1% $LiCH_3COO$, 0.7% agarose, 200V, ~15 min, DNA visualized using SYBR® gold Nucleic Acid Gel Stain under UV illumination) confirmed the presence of products of appropriate size (2.3 kb) which were ligated into P-GEM vector (Promega) by blunt end ligation (1µL vector, 4µL PCR-amplification product, 2µL H2O, 1µL T4 DNA ligase (Invitrogen), 4°C 12h). After overnight ligation, *Escherichia coli* cells (strain DH5α – used for all work with *E. coli*) were transformed with the ligation mixture using DMSO heat shock protocol.[1] 50µL of competent DH5α cells were used for each transformation. These were removed from the -20°C freezer and thawed on ice. 1-10µL of ligation mixture and 0.5µL DMSO was added. The mixture was left on ice for 30 min and then heat shocked for 60-90 sec at 42°C before returning to ice for 2 more minutes. 0.4mL of SOC or LB media (without antibiotics) was added to each reaction tube and the culture was incubated at 37°C with shaking for 45-60 min. Transformed bacteria were plated onto preheated (to 37°C) LB + ampicillin plates (0.1%) and incubated at 37°C overnight (12h).

Colonies visible after overnight growth were isolated and tested by PCR-amplification to confirm the presence of the insert. Pre-mixed PCR-amplification buffer, primers, dNTPs, and taq polymerase were aliquoted into a 96 well plate. Reactants per 20L micro-reaction (one well) were 15.35µL H_2O, 2µL 10X Paq buffer (NEB), 0.4µL dNTP, 1µL of each primer (KTSCIS, KTSCIA, each 1 µM), 0.25µL DNA polymerase (NEB). A sterile toothpick was then touched onto a colony and dipped into a single well. This was repeated for numerous colonies on each plate, PCR-amplification performed (94°C- 4min, 5 x (94°C-20s, 49°C-30s, 72°C-2min30s), 30 x (94°C-20s, 50°C-30s, 72°C-2min30s), 72°C-10min), and the resulting products analyzed by gel electrophoresis (conditions as before). Several colonies with appropriate sized inserts (based on PCR-amplification results) were selected and grown overnight in 3mL liquid culture (LB + 1% ampicillin). Plasmids were harvested from overnight cultures using Qiagen spin columns as directed by the manufacturer.

Several attempts were made to digest the prepared plasmids with both NotI and HindIII (NEB and Invitrogen restriction enzymes were used at various stages in the process). Double digestion was attempted as were sequential digestions in both possible orders. Reaction mixtures were cleaned up between digestions using Qiagen spin columns and small amounts of the reaction mixture periodically analyzed by gel electrophoresis (standard conditions) to gauge reaction progress. In some cases, vectors were linearised, but a product of appropriate size was never obtained.

Further attempts to get the full length sequence into the pYES2 vector were undertaken using blunt-end cloning. Obtaining blunt-ended inserts was not a problem since products from high fidelity PCR-amplification have blunt ends. However, the pYES2 vector did not have a blunt restriction site within its multi-cloning site. This problem was solved by digesting the vector using EcoRI and filling in the hanging ends by using blunt-end polishing (10µL reaction mix containing 1µL Phusion DNA polymerase (NEB), 1 x Phusion PCR-amplification buffer (NEB), 1mM dNTP, 10-500ng DNA, incubated at 72°C, 30 min). The resulting blunt-ended vector and PCR-amplification product were ligated using high concentration ligase (conditions as before) and transformed into *E. coli* (using standard DMSO heat shock protocol) for screening by ampicillin resistance.

Verification of positive clones in the transformants was done by PCR-amplification (procedure as above for screening PCR-amplification). After PCR-amplification, products were analyzed by gel electrophoresis (standard conditions) and positive colonies were then grown in 3mL LB + ampicillin liquid culture overnight. Colonies which were confirmed to contain the insert were also screened with T7 and KTSC1A to confirm directionality. This was possible because the T7 site is located in the plasmid before the insert begins while the KTSC1A site is the reverse primer site within the insert. Overnight cultures were spun down and the plasmids harvested using Qiagen spin columns.

Transformation into lanosterol deficient GIL77 *Saccharomyces cerevisiae* was done using the procedure described by Matt Kaeberlein.[2] Prior to transformation, yeast were maintained on a plate of YEPD media supplemented with henin and ergosterol. Transformed yeast were grown in synthetic complete media without uracil (SC-U) supplemented with henin and ergosterol with glucose as a carbon source. After four days of growth on plates at 28-30°C, any colonies found were transferred to liquid media (SC-U + glucose) for 48 hrs of growth with shaking at 28-30°C and then transferred to SC-U + galactose for 12hrs of induction. After induction, cells were again spun down and transferred to resting medium for 24 hrs.

Cells were harvested by spinning at 4000 rpm for 5 minutes and rinsing twice with distilled deionised water. After the second rinse, cells were spun down again and resuspended in 5mL of 50% ethanol. The mixture was transferred to a small glass vial and 20% KOH added before refluxing for 5 minutes at 70°C to lyse yeast cells. Lipid soluble compounds were extracted with three successive washes of hexane and the resulting solution dried over sodium sulphate. After evaporating hexane under nitrogen gas, samples were dissolved in chloroform. Further analysis was carried out according to standard procedures as described in chapter 2 (pg. 25-26)

A2: Problems encountered

Although I managed several times to obtain viable yeast cultures that appeared to have been transformed (based on survival without supplemental uracil), no difference was ever seen between the lipid soluble extract from the transformed yeast which should

have contained the KTS gene and the yeast transformed with an empty pYES2 vector as a control. The presence and orientation of all inserts was confirmed in pGEM, and some inserts were confirmed to be present in pYES2 and in correct orientation. However, repeated efforts to express these genes in yeast were unsuccessful.

Numerous problems were encountered during the course of this experiment with contamination of yeast cultures, questionable activity of enzymes, and various other factors which hampered progress. It is hoped that future work on characterization of these genes can benefit from my experience in molecular biology attempts and plant wax chemical analyses.

A3: Yeast media preparation details

Following are the details of procedures for preparation of various yeast media used in this experiment. Details of bacterial media preparation are widely available and as such will not be repeated here.

Yeast extract peptone dextrose medium supplemented with hemin chloride and ergosterol (YEPD+HE)

To make 1 L of YEPD+HE, the following amounts of each component were added to 980 mL distilled water, and the solution was autoclaved:

- 1% (10 g/L) yeast extract (Difco, USA)
- 2% (20 g/L) Bacto peptone (Difco)
- 2% (20 g/L) dextrose (EM, Germany)
- 1.5% (15 g/L) Bacto agar (Difco) for plates

After autoclaving, 20 mg/L of ergosterol (Sigma, Canada), first dissolved in 5 mL ethanol and 5 mL Polyoxyethylene (20) sorbitan monooleate (EMD, Germany), was added by sterile filtration. 13 mg/L hemin chloride (Calbiochem, USA), dissolved in 10 mL 50% ethanol with several drops of 5N NaOH was also added by sterile filtration to bring the total volume to 1 L.

Synthetic complete medium without uracil, supplemented with hemin chloride and ergosterol (SC-U+HE)

To make 1 L of SC-U+HE, the following amounts of each component were added to 880 mL distilled water, and the solution was autoclaved:

- 0.01% (100 mg/L) adenine (Alfa Aesar)
- 0.01% (100 mg/L) arginine (Alfa Aesar)
- 0.01% (100 mg/L) cysteine (Alfa Aesar)
- 0.01% (100 mg/L) leucine (Alfa Aesar)
- 0.01% (100 mg/L) lysine (Alfa Aesar)
- 0.01% (100 mg/L) threonine (Alfa Aesar)
- 0.01% (100 mg/L) tryptophan (Alfa Aesar)
- 0.005% (50 mg/L) aspartic acid (Alfa Aesar)
- 0.005% (50 mg/L) histidine (Alfa Aesar)
- 0.005% (50 mg/L) isoleucine (Alfa Aesar)
- 0.005% (50 mg/L) methionine (Alfa Aesar)
- 0.005% (50 mg/L) phenylalanine (Alfa Aesar)
- 0.005% (50 mg/L) proline (Alfa Aesar)
- 0.005% (50 mg/L) serine (Alfa Aesar)
- 0.005% (50 mg/L) valine (Alfa Aesar)
- 0.67% (6.7 g/L) yeast nitrogen base without amino acids (Invitrogen, Canada)
- 2% (20 g/L) Bacto agar (Difco) for plates

After autoclaving, medium was supplemented with hemin chloride and ergosterol in the same manner as was used for YPED+HE. The appropriate carbon source was added by sterile filtration to bring the total volume to 1 L:

2% glucose SC-U+HE for plates and liquid growth: 20 g dextrose (EM) in 100 mL distilled water added by sterile filtration.

2% galactose SC-U+HE for induction of pYES2: 20 g galactose (Sigma) in 100 mL distilled water added by sterile filtration.

Resting medium

To make 1 L of resting medium, the following amounts of each component were added to 990 mL distilled water, and the solution was autoclaved:

- 0.1 M (13.6 g/L) potassium phosphate (EMD)

- 3% (30 g/L) dextrose (EM)

pH was adjusted to 7.0 and this was supplemented with hemin chloride as before, but not ergosterol, to bring to a total volume of 1 L.

References

(1) Chung, C. T.; Niemela, S. L.; Miller, R. H. *Proc. Natl. Acad. Sci. USA* **1989,** *86*, 2172-2175.

(2) Kaeberlein, M. Yeast Transformation. *http://web.mit.edu/biology/guarente/protocols/ytransformation.html* (accessed 09/12, 2006).