Assessing surface wax chemical diversity as a tool to defend against abiotic and biotic stress in canola.

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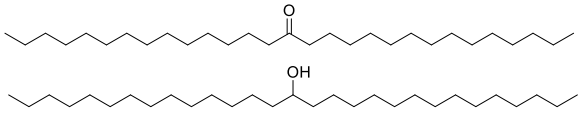

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SaskCanola

Plain language summary

Study: Assessing surface wax chemical diversity as a tool to defend against abiotic and biotic stress in Canola.
Principal investigator: Mark Smith, AAFC Saskatoon
This research is part of the Canola Agronomic Research Program (CARP Grant 2018.18) with funding from the Saskatchewan Canola Development Commission (SaskCanola).

Purpose: The outer surface of a canola plant is covered by a complex mixture of water-repelling material referred to as cuticular wax. This layer plays a role in prevention of water loss from the plant and is also the first thing an insect or fungal spore encounters when landing on a leaf or petal. The purpose of the project is to identify the chemicals that make up this wax and to see if this is the same all-over the plant and between different canola varieties. We are also identifying genes involved in wax biosynthesis and genes that control wax production. The long term goal is to determine how wax composition can be changed through breeding to enhance the ability of the plant to protect itself from drought and attack from insect or fungal pests.

Results: We used modern analytical technologies to identify the natural chemicals which form the surface wax of canola (*Brassica napus*). As wax does not dissolve in water, we washed wax off leaves, stems, pods and flowers with a solvent before analysing it using gas chromatography and mass spectrometry. The wax was found to be a complex mixture of aliphatic hydrocarbons (long chains of carbon and hydrogen), with 5 main components and many minor ones. Wax from different parts of the plant, except for petals, was very similar with only small differences in the proportion of the chemicals. Many chemicals found on leaves were not present on petals. Different canola varieties were analysed, from some of the earliest such as Tower and Westar, to modern hybrid varieties. Most varieties had wax composition very similar to each other suggesting that there is limited chemical diversity in this species, but also that breeding modern varieties has not changed the wax composition to any significant extent. We also examined the wax of species closely related to canola such as Polish rapeseed (*Brassica rapa*) and carinata (*Brassica carinata*). Wax from these plants were chemically similar to canola wax. Although we did not study the function of the wax in detail, we observed that plants in drought stress produce more wax, likely as a way to conserve water. To speed up breeding for useful differences in wax composition it is helpful to know what genes are involved in wax synthesis in the plant. To identify these genes we peeled off the outer layer of the plant, called the epidermis, and used DNA sequencing to identify the genes and generate a list of ones that were active during wax production. Further validation is in progress to determine which genes play the most important roles in determining composition and wax amount.

The work has considerably enhanced our understanding of wax chemistry, diversity and biosynthesis in canola and identified gaps where further knowledge is required. It has established the groundwork for studies probing the role of wax in plant protection and provided information on the target genes that could be useful in breeding for modified wax to address specific challenges.

Project Team.

Project Lead.

Dr Mark Smith. Agriculture and Agri-Food Canada, Saskatoon Research Centre (AAFC-Saskatoon).

Collaborators.

Dr. Sally Vail. AAFC-Saskatoon (Field material, DH12075 *Brassica napus* line).

Dr Steve Robinson. AAFC-Saskatoon.

Dr Isobel Parkin. AACF-Saskatoon (Guidance on transcriptome data processing and genomics, access to *B. napus* genome sequence data).

Dr Miles Buchwaldt. AACF-Saskatoon (Bioinformatics).

Dr Raju Soolanayakanahally. AAFC-Saskatoon (Field grown varieties and abiotic stress lines)

Laboratory Team.

Daniel Hupka. AAFC-Saskatoon (Sample prep, GC and GCMS-analysis).

Helen Lui. AAFC-Saskatoon (Molecular biology).

Laila El-Kalaawy. UVIC CO-OP student (Sample prep and wax analysis, 3 month term, fall 2018).

Claire Stevens. UVIC CO-OP student (Sample prep, method development and wax analysis, 4 month term, summer 2019).

With help from additional students for field work (Ryan, Brooke, Liam and Julia).

Other Resources.

Access to *Brassica carinata* field material provided by Dr Christina Eynck, AAFC-Saskatoon.

Brassica napus historical lines and other species provided by the Plant Gene Resource of Canada (PGRC https://pgrc.agr.gc.ca/index_e.html) and Dr Raju Soolanayakanahally AAFC-Saskatoon.

Analysis.

Gas Chromatography (GC) conducted at AAFC-Saskatoon.

GC-Mass Spectrometry conducted at AAFC-Saskatoon and at the Saskatchewan Structural Sciences Centre, University of Saskatchewan. https://sssc.usask.ca/

SSSC | Saskatchewan Structural Sciences Centre

General introduction.

The outer surfaces of the aerial parts of plants are covered by a complex mixture of water-repelling organic material referred to as cuticular wax. Chemical components of this layer play a fundamental role in the reduction of uncontrolled (non stomatal) water loss from the plant and hence have great importance in drought tolerance (Xue et al 2017). The chemical composition and amount of material present has also been implicated in defense and susceptibility to biotic stresses such as fungal pathogens and insect pests (Muller and Riederer 2005). As examples, the presence of long chain aldehydes, common components of plant waxes, promotes spore germination and cell penetration by powdery mildew in maize (Hansjakob et al 2011). The triterpenes α- and β-amyrin have been shown to deter insect feeding for a number of species and free fatty acids deter the settling of aphids on artificial surfaces and on plants (Eigenbrode and Espelie 1995). In *Brassica napus*, relatively little work has been done to investigate the biological function of cuticular wax. In early studies, leaf wax in *B. napus* conferred partial resistance to infection by *Alternaria brassicae* (blackspot) through a physical mechanism with the wax providing a water-repellant surface and preventing conidia retention (Tewari and Skoropad 1976). Removal of wax increased conidia retention, but also enhanced spore germination suggesting that additional chemical factors were involved (Conn and Tewari 1989). A more recent paper presents evidence for the contribution of wax in the defense of resistant *B. napus* cultivars to sclerotinia infection, although the mechanism for this effect is currently unclear (Ni et al 2014). In vegetable Brassicas, glossy (reduced wax) plants seem to show some resistance to cabbageworm and aphids and less effectively to diamond back moth larvae (Stoner 1990, Eigenbrode and Espelie 1995) whereas in *B. napus* presence of cuticular wax is an important factor determining the rate and feeding pattern of flea beetles on seedlings (Bodnaryk 1992).

Although a clear understanding of the role of wax amount and composition in defense against abiotic and biotic stress has yet to be established, the ability to use genetics and breeding to manipulate wax shows promise as a powerful but overlooked method to boost the ability of canola to defend against pests and diseases and improve drought tolerance. To take advantage of the beneficial chemical properties of cuticular wax it is important to understand the composition of this material. Knowing the chemistry is the key to unlocking and manipulating the genetic pathways controlling wax composition and its regulation. This information will also be important to better understand the interactions between cuticular wax and insect pests and pathogens. Also important is understanding the diversity within *B. napus* and closely related species. Diversity, either natural or induced through mutagenesis or techniques such as genome editing, will enable the process of breeding for the desired wax traits.

Early work conducted more than 40 years ago focused on waxy varieties of *B. oleracea*, including cabbage and Brussels sprouts, with some work on rapeseed type *B. napus*. Major wax components were determined to be long-chain hydrocarbons (alkanes), predominantly nonacosane (a 29-carbon alkane) accompanied by wide range of ketones, secondary alcohols, aldehydes, fatty acids and esters (Macey and Barber 1970, Holloway et al 1977). Minor components including triterpenes (primarily α-amyrin and β-amyrin), ketols and branch chain alkanes have been reported from *B. oleracea* and may also be present in *B. napus* (Martelanc et al 2007, Baker and Holloway 1975). Wax production in B. oleracea appears to be developmentally regulated, and is also responsive to environmental conditions. It remains to be determined if the wax on *B. napus* is uniform in composition throughout the plant, or highly variable, as

is seen in wheat (Tulloch 1973). The role of the environment in regulating wax synthesis is also unclear for *B. napus*. Furthermore, use of the glaucous and glossy phenotypes have long been used in crop breeding and has allowed for the selection of low wax varieties of many vegetable brassicas. This trait has also received some attention in *B. napus* in the context of improving drought tolerance (Pu 2013). The glaucous/glossy phenotype is based on the light scattering properties of wax crystals formed by certain wax components. With increased understanding of the chemical complexity of plant waxes it is now clear that this characteristic is not well suited for phenotyping wax composition as many important compounds in wax are present at relatively low levels or do not form crystals with light scattering properties. Plants with similar glaucous or glossy phenotypes do not necessarily share identical wax composition. In summary, it is clear that we do not yet know enough about wax chemistry, regulation and distribution to fully exploit the potential of this trait.

A genetics based approach to modify cuticular wax requires genetic diversity. In the single diversity study published to date for *B. napus* (Tassone et al 2016), data demonstrated some small variability between cultivars in both total wax load and also in chemical composition, suggesting that phenotypic diversity was influenced primarily by as yet unknown genetic factors. Application of more advanced techniques such as the mapping of quantitative trait loci (QTLs) has not been conducted. The development of improved chemical analysis techniques and a large, extensively genotyped *B. napus* population, in the form of the nested association mapping (NAM) population recently developed at AAFC in Saskatoon, offers a unique opportunity to fully understand cuticular wax composition and begin to realize its potential through breeding.

Project objectives.

The project objectives were aimed at building a unique resource that could act as a foundation for further work towards the development of improved varieties through the manipulation of surface wax in *B. napus*. Objectives were to:

1. Conduct a detailed chemical analysis of the amount, distribution and chemical composition of surface wax on *B. napus* plants and monitor changes with growth and the effect of the environment.
2. Establish the degree of chemical diversity of surface wax within *B. napus* to determine the genetic potential for trait manipulation.
3. Use transcriptome analysis to identify potential genes involved in synthesis and regulation in *B. napus,* thus enabling trait manipulation and the development of robust molecular markers for breeding purposes.
4. Monitor for additional accessible genetic diversity by determining wax composition within the six species of the triangle of U and a subset of RILs from the *Brassica napus* NAM population.
5. Identify *B. napus* lines with clearly defined cuticular wax composition for use in insect and pathogen interaction studies.

Methods.

Wax extraction.

As epicuticular wax is not soluble in water, wax was collected from plant surfaces by washing with a solvent (chloroform, $CHCl_3$). To quantify the amount of wax present, plant parts were measured prior to wax extraction and the wax weighed after recovery to determine wax amount/unit surface area. For some analyses, internal standards (octacosane and nonadecanoic acid, each at 4μg/ml final concentration) were added to the chloroform prior to extracting the plant material. Measured plant parts were gently shaken for 30 seconds in a known volume of chloroform, then removed and dried to determine dry weight. The chloroform extract was filtered to remove debris and died under a gentle steam of nitrogen gas. Dried wax was weighed and the wax was re-dissolved in hexane:toluene (1:1 v/v) to achieve a final concentration of around 20mg/ml. Wax samples were stored at -20 degrees in sealed vials and samples were warmed to redissolve precipitated components prior to analysis.

GC and GC-MS analysis.

For quantitative analysis and to determine percentage composition of the various wax components, samples were analysed by gas chromatography (GC). Analysis was conducted either on an Agilent 6890 GC equipped with a flame ionization detector (FID) and a 30m HP-5 column, with Helium as a carrier gas, or a Thermo Scientific TRACE 1310 GC equipped with a 30m Thermo Scientific TQ-SQC column (30m x 0.25mm x 0.25μm) split to an FID and TQ-MS, with Hydrogen as a carrier gas. To identify wax components by mass spectrometry, samples were separated using the ThermoFisher Trace 1300 GC as above and analysed using a Thermo Scientific TSQ 9000 triple quadruple MS/MS operating in electron ionization (EI) full scan mode. For high accurate mass determination samples were analysed at the Saskatchewan Structural Sciences Centre (University of Saskatoon) using a JOEL AccuTOF GC/MS equipped with a 30m DB5-MS column. Figure 1 illustrates the wax extraction and analysis procedure used in this work.

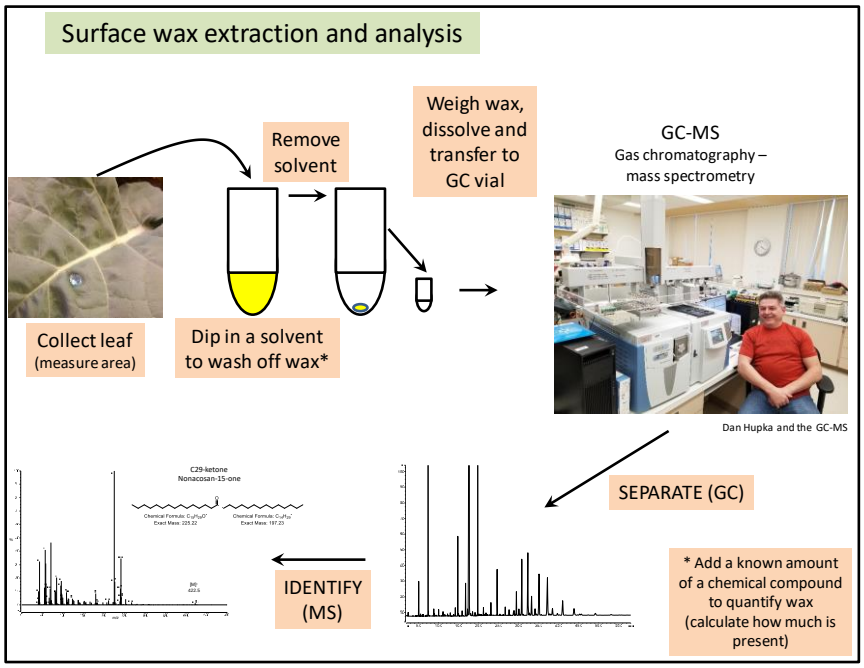


Figure 1. Protocol for surface wax extraction and analysis.

TLC and SPE.

Thin layer chromatography (TLC) for the rapid separation of wax chemical classes was conducted using aluminium backed silica gel G60 plates and a mobile phase of hexane:diethyl-ether:acetic acid (90:7.5:1 v/v/v). Lipid spots were visualized using phosphomolybdic acid stain (PMA). Wax fractionation by solid phase extraction (SPE) was conducted using Sep-Pak Vac 3cc 500mg silica cartridges. Columns were washed extensively with hexane and chloroform prior to use. Samples (10mg) was loaded onto the columns in chloroform and fractions were eluted with solvent mixtures as described in results.

Epidermal and petiole transcriptomes.

Total RNA was prepared from intact petioles and epidermis peeled from young *B. napus* petioles (DH12075) using a Qiagen RNeasy kit. RNA was sent to the National Research Council (NRC) Saskatoon for library preparation and sequencing. Three full-service RNA libraries were prepared for each tissue and sequenced on the Illumina HiSeq2500 (2 x 125 cycles, paired-end reads). Sequences were trimmed to remove adaptors using EdgeR and low quality reads were removed. Reads were aligned to the *Brassica napus* DH12075v3.1 reference genome sequence using STAR. Data was normalized and hits per thousand base pairs were calculated for each gene.

Results.

1. Chemical composition of the surface wax of *Brassica napus*.

To determine the chemical composition of surface wax, the *B. napus* canola quality line DH12075 as chosen for analysis. This line is a doubled haploid line and was the line selected for genome sequencing at AAFC in Saskatoon. Extraction and analysis was conducted as described in the methods section, using greenhouse grown material.

Initial GC analysis showed that the wax was dominated by five major chemical components (Figure 2) accounting for over 90% of the total wax chemicals. These comprised two alkanes, linear hydrocarbons with chain lengths of 29 (C29) and 31 (C31) carbon atoms (nonacosane and hentriacontane), a C29 mid-chain ketone (nonacosan-15-one), a C29 mid-chain (secondary) alcohol (nonacosan-15-ol) and a C30 aldehyde (triacontanal). A large number of minor components were also present (Figure 3) consisting of additional alkanes and aldehydes, primary alcohols, free fatty acids, alkyl-esters and triterpenes. For a more detailed analysis of the minor components, the wax was fractionated by SPE so that components of different chemical classes could be analysed without interference from major compounds with over-lapping retention times on the GC column. Separation of the wax into 5 fractions was achieved (Figure 4) and identities were determined by GC/MS.

Fraction 1 (Figure 5) contained alkanes and showed that although C29 and C31 alkanes were the major components, a wide range of alkanes were present at low levels including the branched chain alkanes C29 *iso*-alkane and C30 *anteiso*-alkane. Chain length ranged from C16 to C33 with both even carbon number and odd carbon number alkanes detected.

Fraction 2 (Figure 6) contained the C29 ketone, aldehydes and alky esters. A total of four aldehydes were detected, all with even carbon chain length from C26 to C32. Additional minor components were also present, but were below the threshold for accurate identification. A significant proportion of this fraction was made up by alkyl esters (also known as wax esters). These esters were present as multiple isomers reflecting synthesis of a complex mixture of linear and branched alcohols and fatty acids, with different combinations esterified to form alkyl esters, often having identical mass. For example the C43 alkyl ester peak on the gas chromatogram was found to be composed of 2 isomers, 27:0-16:0 and 26:0-17:0 (see table 1 for nomenclature).

Fractions 3 and 4 (Figure 7) contained the C29 secondary alcohol nonacosan-15-ol and a contaminant that appeared to be a plasticizer, possibly leached from the plastic of the SPE column. A number of minor components have yet to be identified.

Fraction 5 (Figure 8) contained primary alcohols, with free fatty acids and the triterpenes α-amyrin and β-amyrin being minor components. All of these components could only be identified with confidence after derivatization of the sample using BSTFA to generate TMS-ethers (alcohols and triterpenes) or TMS esters (free fatty acids). Four primary alcohols (hexacosanol (C26), 24-methyl-hexacosanol (C27), octacosanol (C28) and 26-methyl-octacosanol (C29) were identified as major components representing both linear and branched alcohols. Small amounts of other primary alcohols were also detected ranging in chain length from C22 to C30. Levels were too low for accurate quantification. The four fatty acids identified were all even chain linear saturated fatty acids, two long

chain fatty acids (palmitic acid, 16:0, stearic acid, 18:0), and two very-long-chain fatty acids (octacosanoic acid, 28:0, and triacontanoic acid, 30:0).

Our comprehensive analysis showed that although *B. napus* surface wax is dominated by only 5 major components, it is chemically complex with a large number of minor constituents. We identified components not previously reported from *B. napus,* and were able to show that a putative diketone observed previously (Tassone et al 2016) was misidentified (Figure 9) indicating that, unlike wheat, *B. napus* does not have the ability to make this class of wax components. Our results indicated that the routine quantification of all of these components is likely not possible using standard GC methodology due to retention time overlap and masking of minor components. As shown in figure 10 for the variety "Tower" quantitative identification of only around 30 components is possible by GC of underivatized wax in a single step analysis. This represents almost all of the major classes of wax chemicals, although the triterpenes could not be reliably quantified. High throughput screening for minor components would therefore be technically challenging even using a tandem mass spectrometry approach due to the lack of diagnostic fragment ions in these chemically simple compounds. A list of all of the chloroform soluble components detected in *B. napus* wax is given in table 1. A library of EI (electron ionization) mass spectra has been assembled for easy identification in subsequent work (Available on request).

Figure 2.

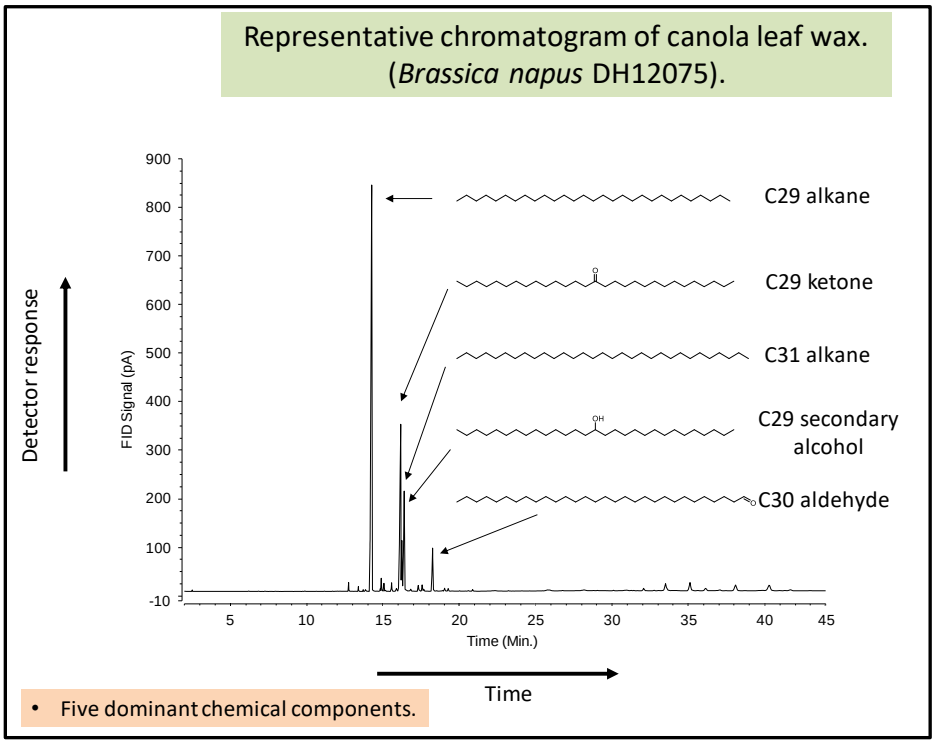


Figure 3.

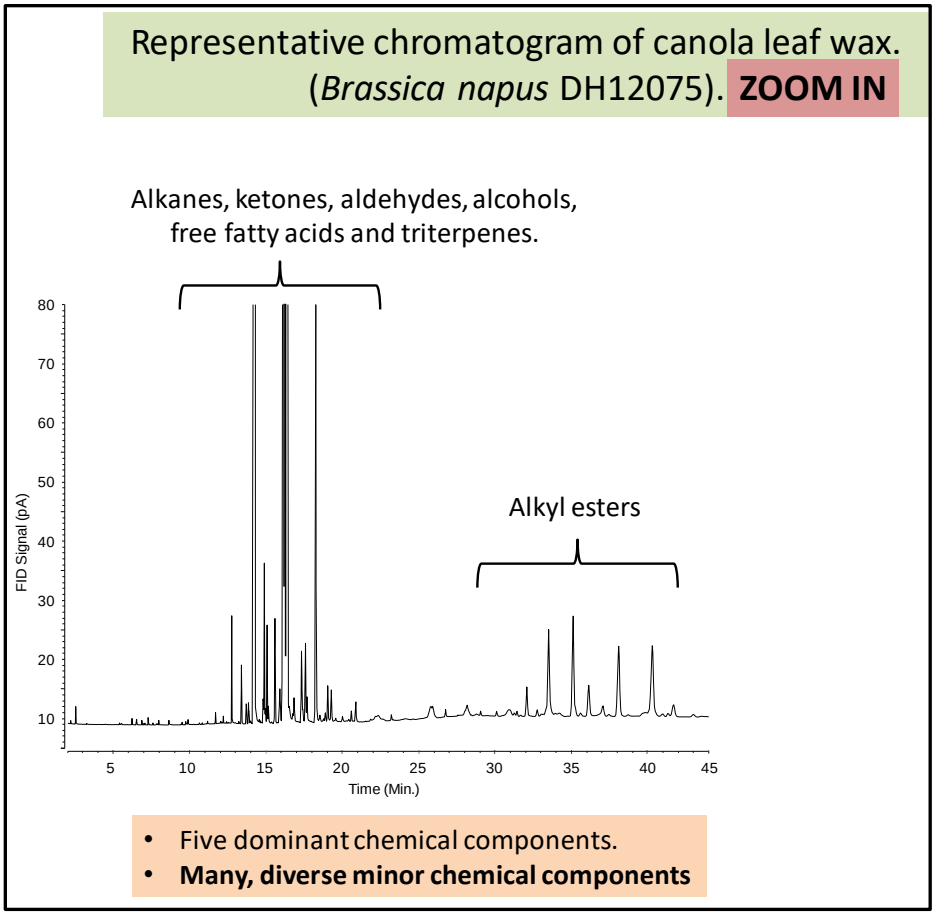


Figure 4. TLC showing separation of wax component classes achieved using SPE.

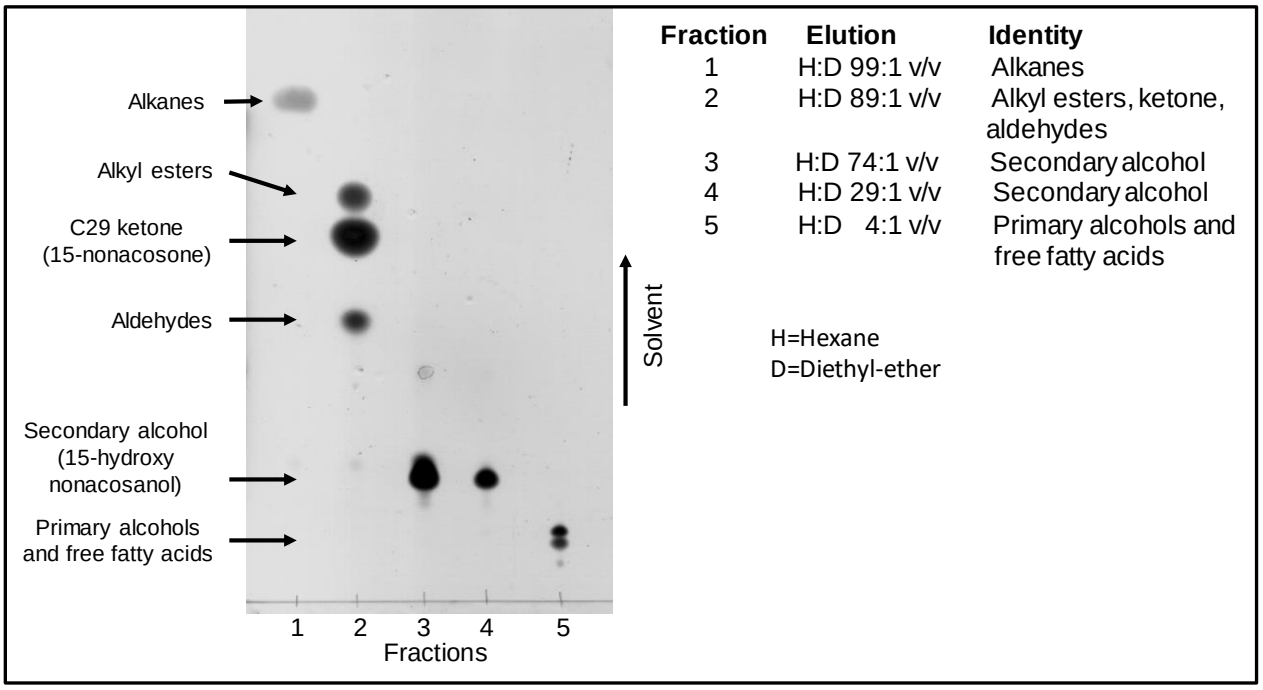


Figure 5.

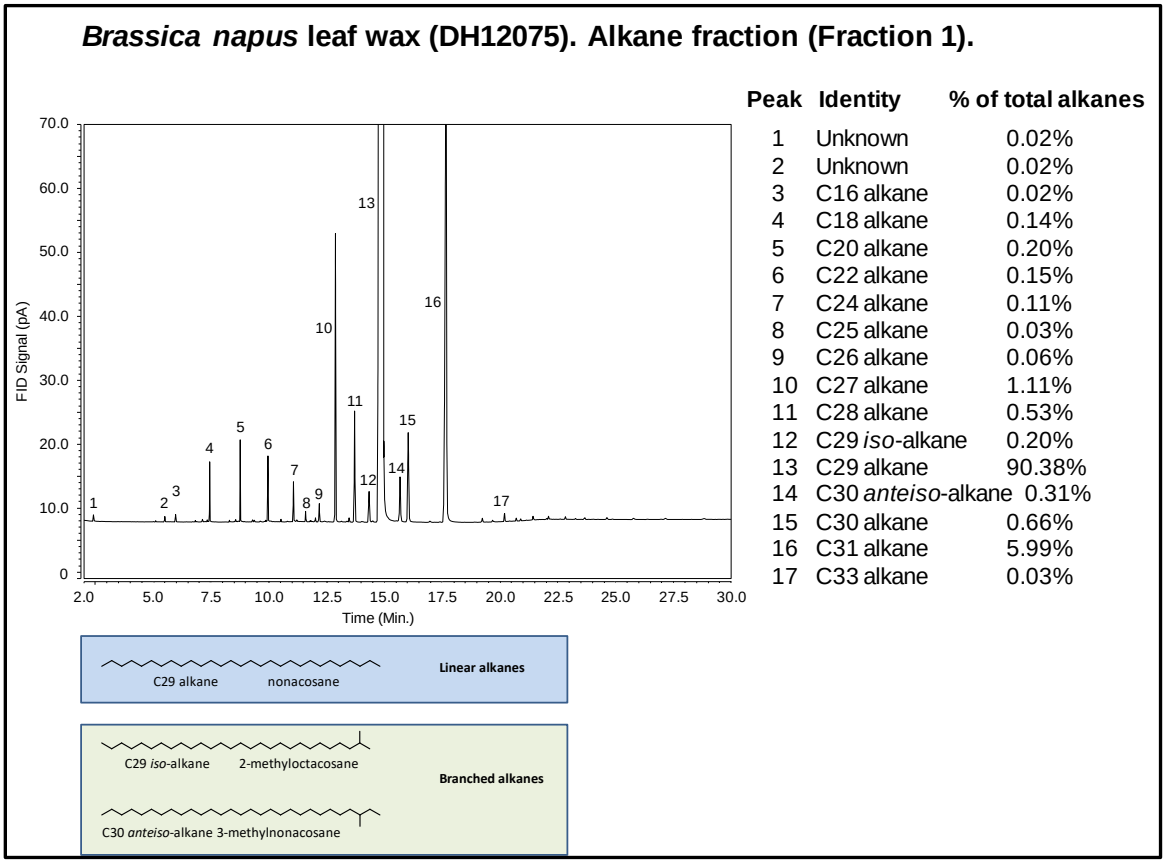
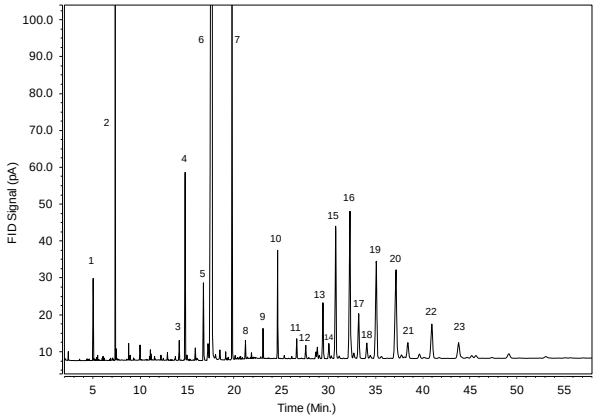


Figure 6

***Brassica napus* leaf wax (DH12075). Ketone, Aldehyde and Alkyl ester fraction. (Fraction 2)**



Peak	Identity	% of total fraction
1	Contaminant	
2	Contaminant	
3	C26 aldehyde	0.24
4	C29 alkane	2.31
5	C28 aldehyde	1.42
6	C29 ketone	60.35
7	C30 aldehyde	4.70
8	C32 aldehyde	0.13
9	C36 alkyl ester	0.30
10	C38 alkyl ester	1.49
11	C40 alkyl ester	0.40
12	C41 alkyl ester	0.29
13	C42 alkyl ester	1.54
14	C42 alkyl ester	0.42
15	C43 alkyl ester	4.34
16	C44 alkyl ester	5.72
17	C44 alkyl ester	1.77
18	C45 alkyl ester	0.63
19	C45 alkyl ester	4.75
20	C46 alkyl ester	5.18
21	C46 alkyl ester	0.81
22	C47 alkyl ester	2.06
23	C48 alkyl ester	1.15

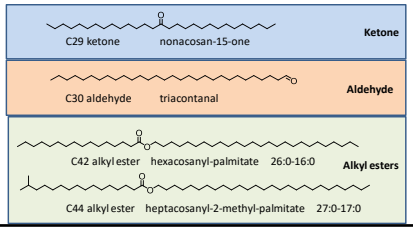
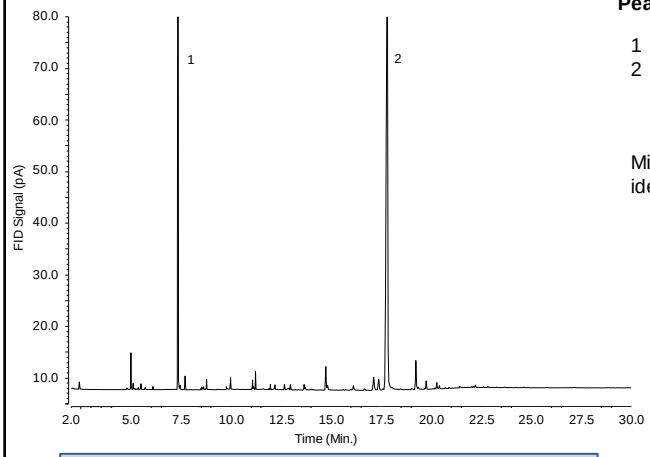


Figure 7.

***Brassica napus* leaf wax (DH12075). Secondary alcohol fraction (Fractions 3 and 4).**



Peak	Identity
1	Contaminant
2	C29 secondary alcohol Nonacosan-15-ol

Minor components could not be identified with confidence.

Figure 8.

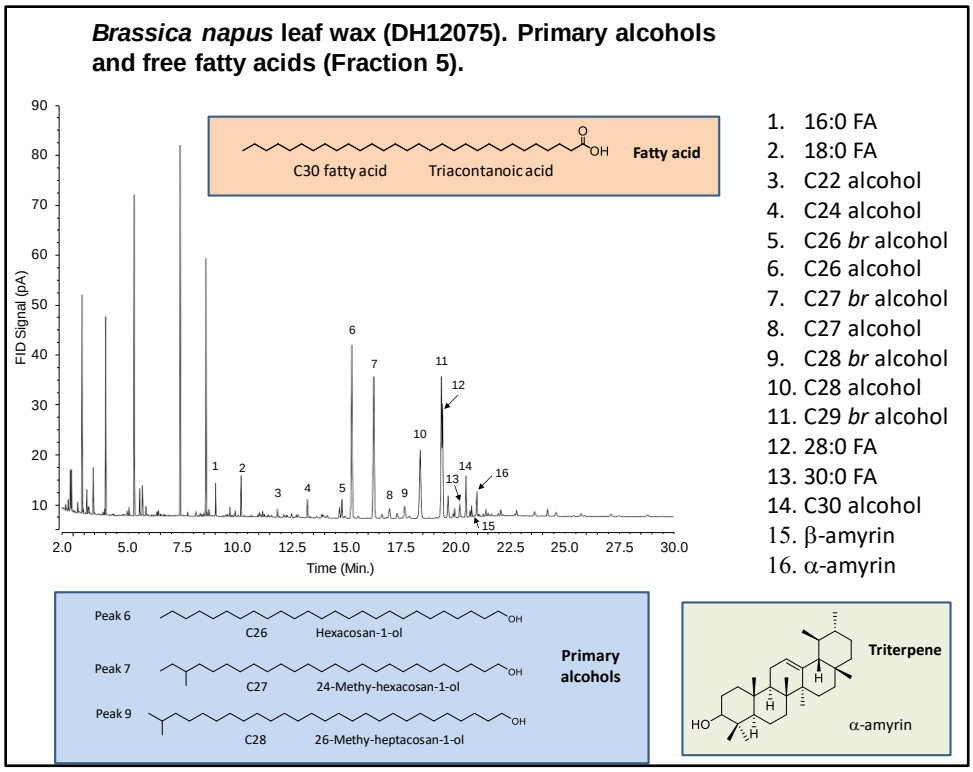


Figure 9. Mass spectra of nonacosan-15-ol

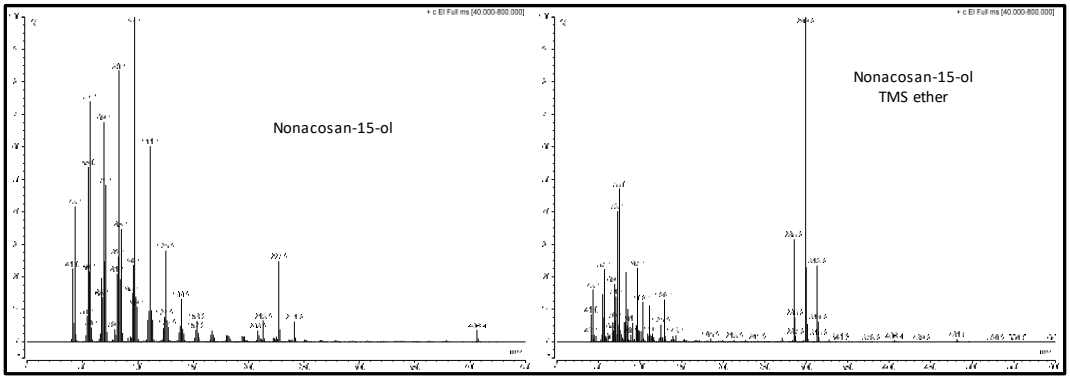
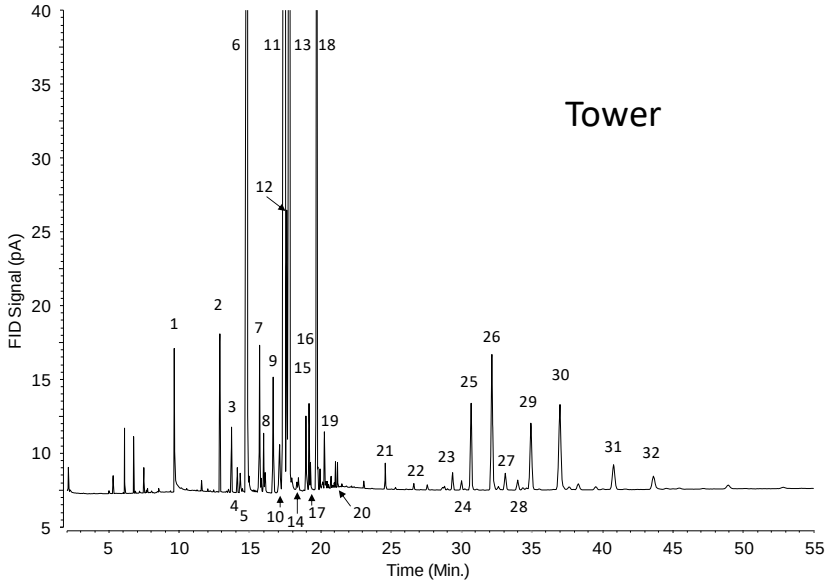


Table 1. Chloroform soluble components of *Brassica napus* wax.

Class	Chain length	Name		Class	Chain length	Name
Alkanes	C16	Hexadecane		**Free fatty acids**	C16	Palmitic acid
	C18	Octadecane			C18	Stearic acid
	C20	Eicosane			C28	Octacosanoic acid
	C22	Docosane			C30	Triacontanoic acid
	C24	Tetracosane		**Alkyl esters**	C36	Multiple isomers
	C25	Pentacosane			C38	
	C26	Hexacosane			C40	
	C27	Heptacosane			C41	
	C28	Octacosane			C42	
	C29 *iso*	2-Methyl octacosane			C43	
	C29	Nonacosane			C44	
	C30 *anteiso*	3-Methyl nonacosane			C45	
	C30	Triacontane			C46	
	C31	Hentriacontane			C47	
	C33	Tritriacontane			C48	
Aldehydes	C26	Hexacosanal		**Triterpenes**	α-amyrin	
	C28	Octacosanal			β-amyrin	
	C30	Triacontanal				
	C32	Dotriacontanal				
Ketone	C29	Nonacosan-15-one				
Secondary alcohol	C29	Nonacosan-15-ol				
Primary alcohols	C22	Docosan-1-ol				
	C24	Tetracosan-1-ol				
	C26 *iso*	24-Methyl-pentacosan-1-ol				
	C26	Hexacosan-1-ol				
	C27 *anteiso*	24-methyl-hexacosan-1-ol				
	C27	Heptacosan-1-ol				
	C28 *iso*	26-Methyl-heptacosan-1-ol				
	C28	Octacosan-1-ol				
	C29 *anteiso*	26-Methyl-octacosan-1-ol				
	C30	Triacontan-1-ol				

Figure 10



1. Unknown (likely from plastic)
2. C27 alkane
3. C28 alkane
4. C26 aldehyde
5. C29 *iso*-alkane
6. C29 alkane
7. C26 alcohol + C30 *anteiso*-alkane
8. C30 alkane
9. C28 aldehyde
10. C27 alcohol
11. C29 ketone
12. C31 alkane
13. C29 secondary alcohol
14. C28 alcohol
15. C29 alcohol
16. Unknown
17. C28 fatty acid
18. C30 aldehyde
19. C30 fatty acid
20. C32 aldehyde
21. C38 alkyl-ester
22. C40 alkyl-ester
23. C42 alkyl-ester
24. C42 alkyl-ester
25. C43 alkyl-ester
26. C44 alkyl-ester
27. C44 alkyl-ester
28. C47 alkyl-ester
29. C47 alkyl-ester
30. C46 alkyl-ester
31. C47 alkyl-ester
32. C48 alkyl-ester

2. Compositional uniformity of wax on *Brassica napus* surfaces.

The chemical composition of surface wax in wheat is non-uniform, with major differences between stem and leaf (Tulloch 1973). To assess chemical uniformity in *B. napus*, wax was extracted from leaf 6, pod (mid maturity) stem and flower of greenhouse grown DH12075. As shown in figure 11, profiles of all tissues except flower appeared similar with 5 major components present. Wax composition of leaf flower and pod is given in table 2. As flower is a composite plant part we further divided this tissue to petals, anthers and "rest". The wax composition of petals differed significantly from other parts of the plant comprising mostly alkanes (Figure 12) with alkyl esters, aldehydes, primary alcohols and the C29 ketone and secondary alcohols being of low abundance or absent.

Figure 11.

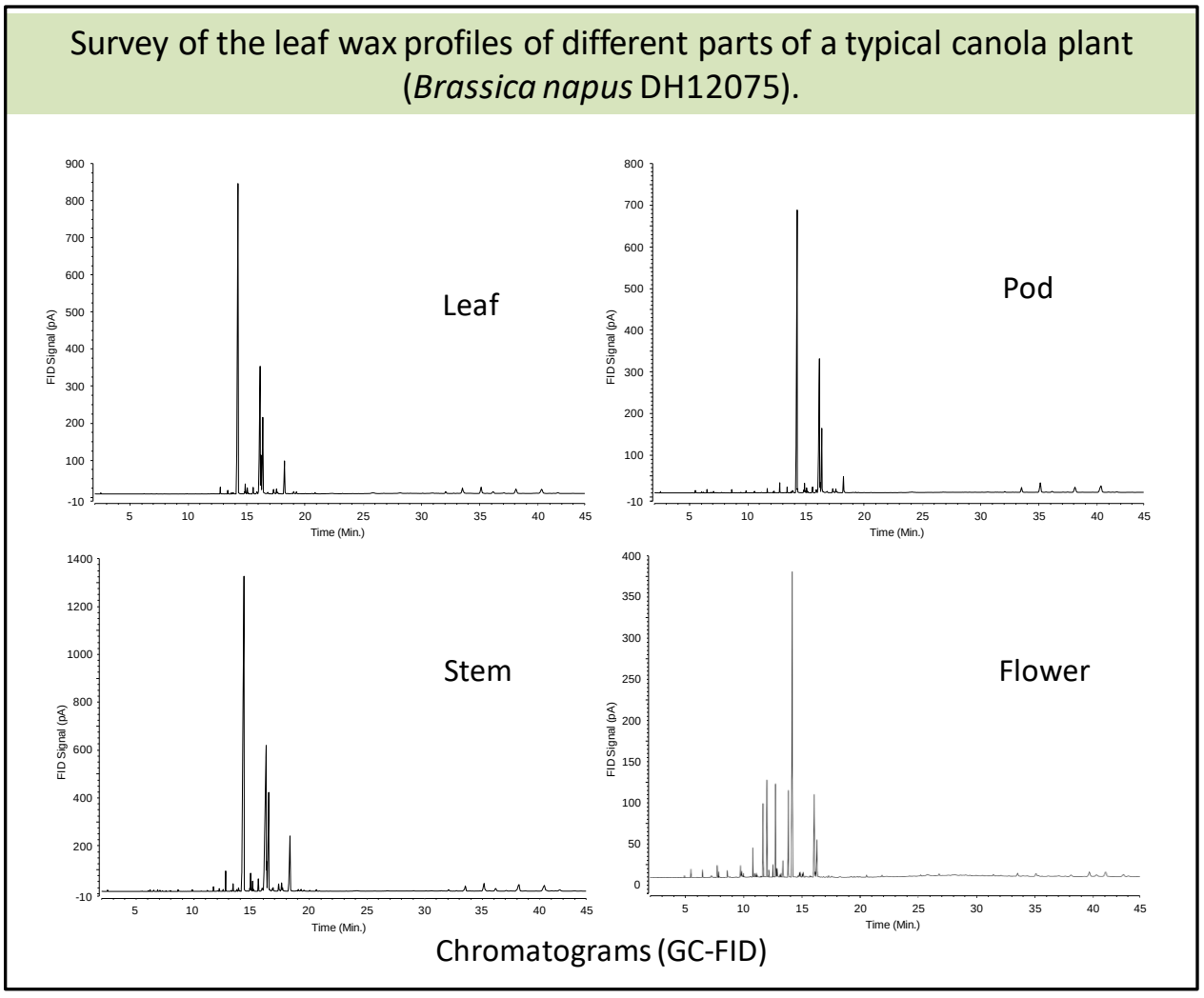


Figure 12.

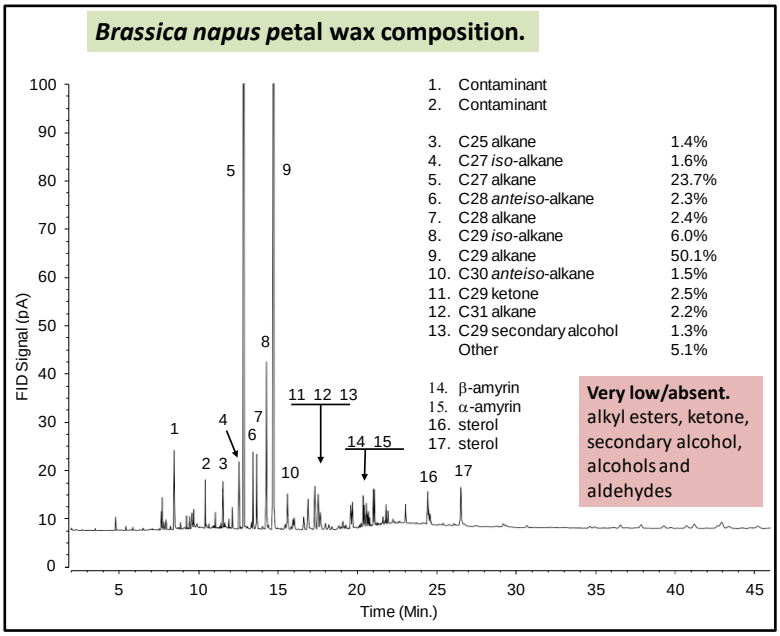


Table 2. Wax composition by percentage leaf, stem and pod of *B. napus* DH12075.

	Leaf	Stem	Pod
C27 alkane	0.33	0.58	0.54
C28 alkane	0.22	0.28	0.38
C26 aldehyde	0.10	0.13	0.18
C29 *iso*-alkane	0.06	0.12	0.15
C29 alkane	44.89	44.46	44.47
C26 alcohol + C30 *anteiso*-alkane	0.81	0.96	0.94
C30 alkane	0.55	0.56	0.47
C28 aldehyde	0.62	0.74	0.62
C27 alcohol	0.33	0.39	0.51
C29 ketone	21.64	24.74	24.5
C31 alkane	4.75	2.25	1.16
C29 alcohol-2	10.07	8.12	9.16
C28 alcohol	0.12	0.07	0.17
C29 alcohol	0.63	0.37	0.54
Unknown	0.58	0.66	0.50
C28 fatty acid	0.21	0.63	0.16
C30 aldehyde	4.08	5.77	2.21
C30 fatty acid	0.28	0.12	0.09
C32 aldehyde	0.24	0.14	T
C42 alkyl ester	0.38	0.18	0.20
C43 alkyl ester	1.50	0.75	1.35
C44 alkyl ester	1.76	1.43	2.99
C44 alkyl ester	0.57	0.52	0.33
C45 alkyl ester	1.51	1.53	2.02
C46 alkyl ester	1.72	1.57	2.9
C46 alkyl ester	T	0.3	T
C47 alkyl ester	0.75	0.85	1.16
Total alkanes	**50.80**	**48.25**	**47.17**
Total aldehydes	**5.04**	**6.78**	**3.01**
Total alkyl esters	**8.47**	**7.13**	**10.95**

3. Survey of wax composition of historical *Brassica napus* varieties.

Brassica napus has undergone extensive selective breeding to develop elite canola quality varieties. To assess the effect of breeding on surface wax, 2 studies were conducted, one using a small number of greenhouse grown lines and one using field grown materials. For the first study, 10 open pollinated varieties of *B. napus* were grown in the greenhouse along with an early polish (*B. rapa*) variety Bronowski. Registration dates ranged from 1968 (Oro) to 1999 (Nex 700). As shown in table 3 wax composition was similar for all varieties, but with some variation in relative percentage of individual components. For example, the C31 alkane ranged from 1.09% of total lipid compounds in Midas to 6.96% in Reston. Large differences in the wax amount were observed between the varieties. The results indicate that the development of canola over the time period analysed likely did not result in major changes to the wax profile. For confidence, additional lines, spanning 1966 to 2019 were sampled from field grown plants (Vail and Soolanayakanahally) for comparison in the field during the 2019 season, with analysis of leaf material at a similar stage to the previous greenhouse study. The results, summarized in table 4 again indicated relatively small differences in percentage composition between the lines. Two varieties 74-44BL and 75-42CR showed higher than average total alkane content, with a corresponding reduction in C29 ketone. Line YN04-C1213 appeared to have a relatively low level of alkyl esters. Comparison of 5 lines grown both in the greenhouse and field showed small but consistent differences in wax composition depending on the environment (Table 5). In all 5 lines, greenhouse grown lines had higher percentage of total alkyl esters and C29 ketone, and lower levels of C29 alkane, as illustrated for AC elect in figure 13.

Table 3. Wax composition of greenhouse grown *B. napus* varieties and *B.rapa* (Bronowski) varieties.

	Oro	Midas	Tower	Altex	Andor	Reston	AC Excel	AC Elect	Nex-700	Bronowski	Ghobi Saron	Average	Range	
Registration year	**1968**	**1973**	**1974**	**1978**	**1981**	**1982**	**1990**	**1992**	**1999**				**Low**	**High**
Wax load (mg/cm^2)	1.89	1.86	3.32	0.55	0.73	2.71	2.11	2.30	2.19	1.41	1.23	**1.85**	0.55	3.32
C27 alkane	0.55	0.39	0.5	0.74	0.42	0.51	0.36	0.4	0.6	0.33	0.44	**0.50**	0.36	0.74
C28 alkane	0.3	0.24	0.27	0.36	0.22	0.23	0.19	0.21	0.26	0.18	0.22	**0.25**	0.19	0.36
C26 aldehyde	T	T	0.11	0.16	0.13	0.12	0.12	0.14	0.13	0.08	0.14	**0.13**	0	0.16
C29 *iso* alkane	0.14	0.13	0.15	0.18	0.19	0.15	0.17	0.19	0.12	0.16	0.22	**0.16**	0.12	0.19
C29 alkane	50.1	48.15	46.02	44.81	43.43	43.21	47.49	46.15	46.21	42.87	40.91	**46.17**	43.21	50.1
C26 alcohol + C30 *anteiso* alkane	0.81	0.71	0.71	1.01	1.47	0.7	1.05	0.77	0.67	1.26	1.05	**0.88**	0.67	1.47
C30 alkane	0.41	0.38	0.36	0.59	0.44	0.54	0.34	0.35	0.29	0.4	0.53	**0.41**	0.29	0.59
C28 aldehyde	0.75	0.54	0.77	1.06	1.02	0.85	0.9	0.94	0.96	0.67	0.93	**0.87**	0.54	1.06
C27 alcohol	0.48	0.55	0.49	0.46	0.55	0.43	0.46	0.53	0.52	0.38	0.45	**0.50**	0.43	0.55
C29 Ketone	22.52	24.51	23.66	21.33	21.71	20.98	22.25	22.82	23.56	21.04	22.89	**22.59**	20.98	24.51
C31 Alkane	2.09	1.09	1.91	5.02	4.43	6.96	1.74	1.83	1.87	4.88	4.32	**2.99**	1.09	6.96
C29 alcohol-2	9.75	9.19	11.35	10.35	8.99	9.81	10.28	10.34	10.66	10.75	10.11	**10.08**	8.99	11.35
C28 alcohol	T	0.16	0.11	0.11	0.13	0.13	0.1	0.1	0.08	0.1	0.13	**0.12**	0.08	0.16
C29 alcohol	0.43	0.48	0.5	0.45	0.72	0.44	0.58	0.6	0.52	0.71	0.51	**0.52**	0.43	0.72
Unknown	0.36	0.53	0.48	0.43	0.55	0.44	0.44	0.48	0.52	0.46	0.6	**0.47**	0.36	0.53
C28 FA	0.15	0.15	0.16	0.2	0.21	0.18	0.21	0.18	0.19	0.24	0.22	**0.18**	0.15	0.21
C30 Aldehyde	2.27	2.01	2.27	2	3.13	2.41	4.26	3.97	2.45	3.09	3.81	**2.75**	2	4.26
C30 FA	0.24	0.23	0.26	0.3	0.33	0.33	0.33	0.32	0.34	0.4	0.41	**0.30**	0.23	0.34
C32 Aldehyde	T	T	0.09	T	T	0.27	T	T	0.09	0.11	0.14	**0.15**	0	0.27
C38 alkyl ester	0.31	T	0.15	0.45	0.29	1.31	0.88	0.4	0.19	0.5	0.79	**0.50**	0.15	1.31
C40 alkyl ester	T	T	0.19	0.13	0.08	0.31	0.2	0.12	0.07	0.13	0.18	**0.16**	0.07	0.31
C42 alkyl ester	0.22	0.15	0.19	0.29	0.18	0.37	0.25	0.25	0.28	0.26	0.25	**0.24**	0.15	0.37
C42 alkyl ester	T	0.09	0.11	0.11	0.12	T	0.1	0.11	0.12	0.1	0.12	**0.11**	0	0.12
C43 alkyl ester	0.98	0.95	1.06	1.13	1.12	1.02	0.96	1.03	1.2	1.17	1.02	**1.05**	0.95	1.13
C44 alkyl ester	1.85	2.14	2.01	1.76	2.45	1.27	1.36	1.55	1.96	1.97	1.51	**1.82**	1.27	2.45
C44 alkyl ester	0.3	0.2	0.25	0.44	0.24	0.52	0.3	0.32	0.3	0.32	0.28	**0.32**	0.2	0.52
C45 alkyl ester	0.16	0.14	0.17	0.2	0.21	0.14	0.16	0.18	0.15	0.16	0.19	**0.17**	0.14	0.21
C45 alkyl ester	1.1	1.16	1.22	1.35	1.28	1.42	1.03	1.19	1.08	1.28	1.11	**1.20**	1.03	1.42
C46 alky ester	1.56	1.96	1.84	1.69	2.22	1.42	1.27	1.56	1.57	1.69	1.47	**1.68**	1.27	2.22
C46 alkyl ester	0.17	0.11	0.13	0.23	0.13	0.31	0.16	0.17	0.16	0.16	0.14	**0.17**	0.11	0.31
C47 alkyl ester	0.57	0.61	0.59	0.65	0.63	0.68	0.55	0.61	0.52	0.59	0.54	**0.60**	0.52	0.68
C48 alkyl ester	0.28	0.39	0.34	0.3	0.45	0.28	0.23	0.35	0.26	0.37	0.31	**0.32**	0.23	0.45
Total alkyl esters	7.5	7.9	8.25	8.73	9.4	9.05	7.45	7.84	7.86	8.7	7.91	**8.24**	7.45	9.4
Total alkanes	53.59	50.38	49.21	51.7	49.13	51.6	50.29	49.13	49.35	48.82	46.64	**49.99**	46.64	53.59
Total aldehydes	3.02	2.55	3.24	3.22	4.28	3.65	5.28	5.05	3.63	3.95	5.02	**3.90**	2.55	5.28

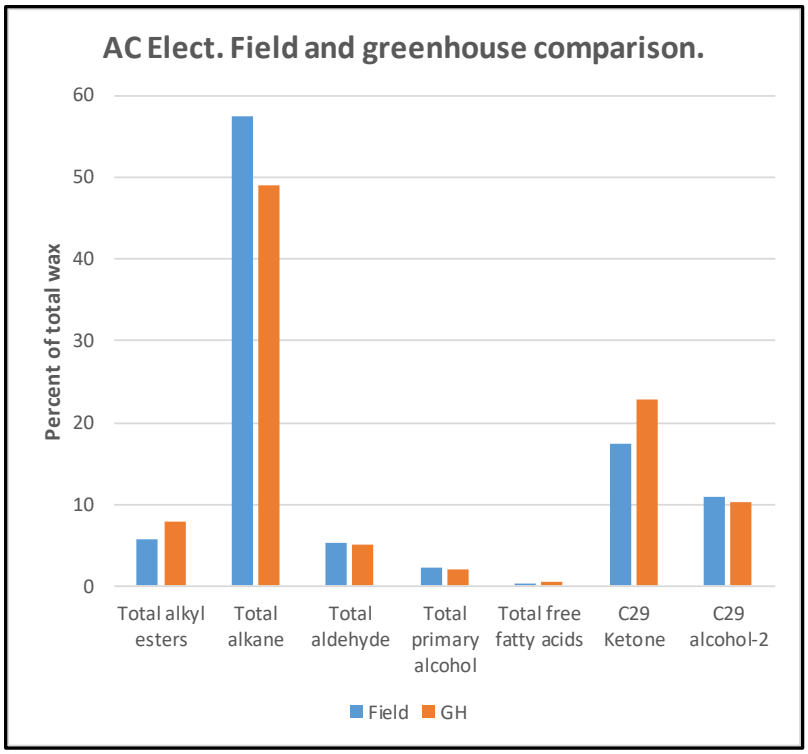
Table 4. Wax composition (summary) of field grown *B.napus* varieties.

Variety	Year of Canadian registration	Total alkyl esters	Total alkanes	Total aldehydes	Total primary alcohol	Total free fatty acids	C29 ketone	C29 alcohol-2
Target	1966	6.24	58.33	4.26	2.06	0.49	17.07	10.67
Oro	1968	6.75	57.47	4.13	1.72	0.39	19.16	10.35
Midas	1973	6.40	57.95	4.21	1.74	0.36	17.88	10.82
Tower	1974	6.76	55.27	4.39	1.94	0.39	19.11	11.43
Westar	1982	6.67	56.00	4.88	2.09	0.49	17.95	11.10
Profit	1989	6.37	56.54	4.95	1.89	0.54	18.68	10.09
AC Excel	1990	5.40	57.34	5.06	2.18	0.47	17.93	10.89
AC Elect	1992	5.63	57.42	5.22	2.25	0.38	17.44	10.84
Defender	1994	6.74	54.21	5.39	2.26	0.56	19.60	10.51
46A65	1996	5.61	55.33	4.24	2.15	0.48	19.89	11.41
Q2	1998	5.97	56.36	4.28	1.88	0.31	19.24	11.35
SP BANNER	2002	4.99	57.33	4.16	2.08	0.33	19.41	11.32
InVigor 5440	2007	6.20	56.97	4.09	1.65	0.50	17.76	11.76
46H75	2011	5.12	54.83	5.07	2.49	0.54	19.75	11.37
74-44BL	2012	5.63	**60.40**	4.91	1.72	0.41	**15.14**	10.99
InVigor L252	2013	5.20	58.02	4.75	1.94	0.31	18.62	10.50
45H76	2014	5.99	54.64	4.04	1.87	0.45	20.13	11.65
PV 200 CL	2015	5.82	52.95	4.60	2.74	0.52	20.52	11.18
PV533G	2015	6.28	54.79	5.65	2.09	0.56	17.69	11.26
InVigor L233P	2016	5.47	56.59	5.45	2.66	0.55	17.70	10.70
75-42CR	2019	5.03	**63.69**	4.98	1.83	0.39	**13.24**	10.34
Argentine		6.78	56.40	4.38	2.00	0.50	18.28	10.82
H151797		5.31	57.50	4.94	2.07	0.38	18.28	10.78
NAM-0		6.14	57.15	4.10	1.78	0.45	17.69	11.94
YN04-C1213		**3.50**	56.27	4.48	2.63	0.58	19.09	12.29

Table 5. Comparison of field and greenhouse grown *B. napus* variety wax profiles.

	Variety	Total alkyl esters	Total alkane	Total aldehyde	Total primary alcohol	Total free fatty acids	C29 Ketone	C29 alcohol-2
Field	**Oro**	6.75	57.47	4.13	1.72	0.39	19.16	10.35
GH	**Oro**	7.5	53.59	3.02	1.72	0.39	22.52	9.75
Field	**Midas**	6.40	57.95	4.21	1.74	0.36	17.88	10.82
GH	**Midas**	7.9	50.38	2.55	1.9	0.38	24.51	9.19
Field	**Tower**	6.76	55.27	4.39	1.94	0.39	19.11	11.43
GH	**Tower**	8.25	49.21	3.24	1.81	0.42	23.66	11.35
Field	**AC Excel**	5.40	57.34	5.06	2.18	0.47	17.93	10.89
GH	**AC Excel**	7.45	50.29	5.28	2.19	0.54	22.25	10.28
Field	**AC Elect**	5.63	57.42	5.22	2.25	0.38	17.44	10.84
GH	**AC Elect**	7.84	49.13	5.05	2	0.5	22.82	10.34

Figure 13. comparison of wax profiles for field and greenhouse grown *B. napus* (AC Elect).



3. Survey of wax diversity in *Brassica napus* varieties.

A number of different analyses were undertaken to explore chemical diversity in *B. napus* surface wax. As plants in each data set were growing in different years, and different conditions (greenhouse vs field) each data set will be considered separately. Comparison of lines within the data set gives useful information on diversity.

Set 1. *Brassica napus* 54 NAM founder lines, field grown in Saskatoon in Summer 2018. Wax was collected from leaf, with at least 3 replicates per line, and analysed by Liquid Chromatography Mass Spectrometry (LC/MS) at Rothamsted Research in England. In agreement with our composition study, four major components were observed, C29 alkane, C29 ketone C29 secondary alcohol and C31 alkane. The C30 aldehyde co-chromatographed with the C29 alcohol. Wax chemical composition was similar between lines, with variation in relative percentage (Table 6), but no lines showing an absence of components of any chemical class, indicating relatively limited chemical diversity in the collection.

LC/MS was not used for further work as the technique was not able to detect and quantify alkyl esters, and not all of the compounds could be identified.

Set 2. Eleven greenhouse grown historical lines. Described above

Set 3. Twenty five field grown historical lines. Described above.

Within the 90 varieties examined, variation appears relatively limited. Only one line was discovered with a significantly different wax chemical profile. Wax collected from leaf, pod and petiole of greenhouse grown plants had very low levels of C29 ketone and C29 secondary alcohol and a correspondingly higher level of C29 alkane (Figure 14).

Figure 14. Wax composition of the *B.napus* high alkane line.

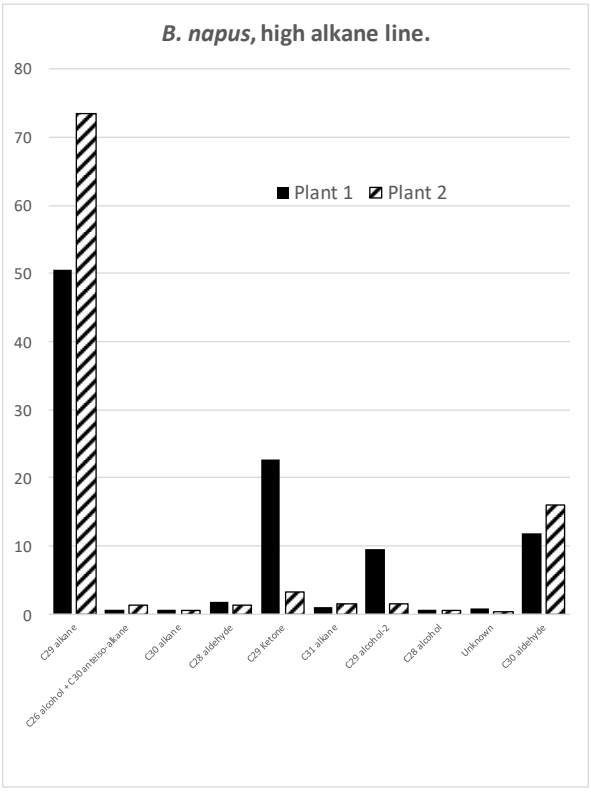


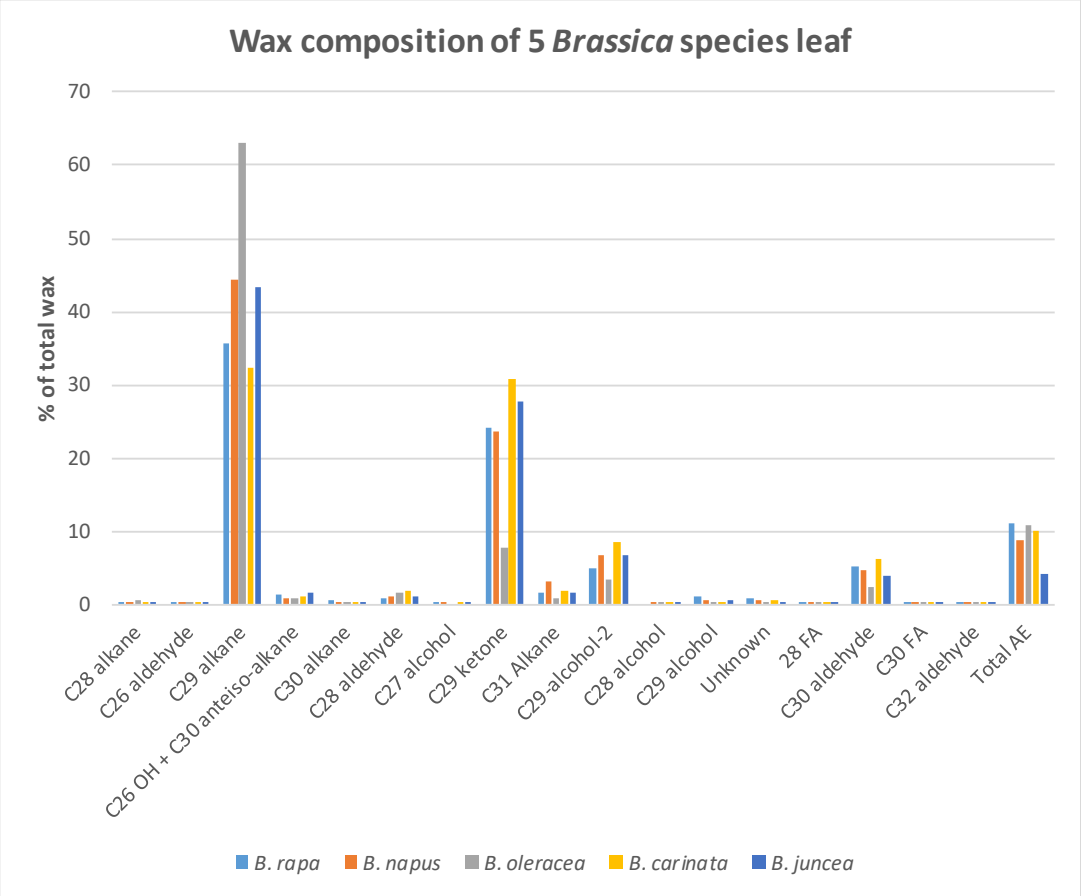
Table 6. Summary of leaf wax composition determined for field grown *B. napus* NAM founder lines.

	Total Alkanes	Total aldehydes	Total primary alcohols	Total free fatty acids	C29 ketone	C29 alcohol-2	Total triterpenes	
NAM-0	54.90	0.70	6.15	1.41	16.86	12.17	5.88	
NAM-1	56.51	1.16	6.40	1.85	15.28	10.16	6.60	
NAM-4	57.82	1.06	7.45	0.75	14.56	10.75	5.34	
NAM-5	42.69	1.98	8.81	5.81	18.09	9.27	10.40	
NAM-10	51.40	1.18	6.40	0.68	15.91	14.86	6.83	
NAM-12	50.20	1.11	7.49	1.57	18.68	11.97	6.54	
NAM-13	56.85	0.95	5.00	0.87	15.61	11.97	6.16	
NAM-14	50.11	1.24	8.72	2.19	14.03	14.11	7.29	
NAM-15	50.86	1.50	6.29	3.60	17.54	10.68	6.86	
NAM-17	50.35	1.84	7.70	3.02	15.97	10.78	7.47	
NAM-23	50.81	1.60	8.35	1.34	16.22	12.45	6.85	
NAM-25	60.28	1.23	5.80	1.14	14.28	10.35	5.56	
NAM-26	56.35	0.91	6.77	1.47	13.12	12.67	5.75	
NAM-28	51.23	2.00	10.43	2.94	13.43	9.65	7.78	
NAM-29	55.61	1.37	7.78	3.77	12.13	10.57	7.31	
NAM-30	51.75	1.24	6.31	2.29	18.65	10.81	6.70	
NAM-31	52.38	0.92	6.17	2.20	17.37	11.23	6.67	
NAM-32	54.82	1.20	6.41	1.83	15.31	10.93	6.45	
NAM-33	51.34	1.58	6.67	3.75	15.09	10.45	8.03	
NAM-34	52.25	0.73	5.55	2.78	17.27	11.06	6.91	
NAM-36	53.23	2.02	7.25	1.86	16.34	9.58	6.32	
NAM-37	54.91	1.55	5.76	1.15	19.30	10.40	5.38	
NAM-38	55.82	1.02	6.48	2.49	15.97	10.40	5.46	
NAM-39	57.61	0.87	8.18	1.34	13.22	11.48	5.25	
NAM-40	52.11	1.41	5.26	1.83	18.53	11.52	6.96	
NAM-42	51.76	1.50	4.95	1.61	19.48	11.92	6.70	
NAM-43	56.00	1.04	4.95	1.39	16.88	11.16	6.13	
NAM-45	50.86	0.43	4.54	2.59	22.07	11.10	5.65	
NAM-46	54.03	0.77	5.63	4.12	16.34	10.66	5.82	
NAM-47	52.39	1.29	7.57	2.51	17.13	11.34	5.94	
NAM-48	52.14	1.95	6.78	1.74	15.75	10.97	7.03	
NAM-49	54.25	0.80	5.95	1.60	14.64	13.54	6.75	
NAM-51	54.41	1.50	8.23	1.22	14.52	10.89	6.67	
NAM-53	55.19	1.28	6.11	4.16	15.16	8.37	6.35	
NAM-56	52.37	1.27	6.08	1.13	17.14	12.39	7.47	
NAM-57	55.40	0.99	5.41	1.42	17.55	11.26	5.71	
NAM-65	52.94	2.46	6.90	1.03	15.94	11.07	7.07	
NAM-66	52.42	1.64	6.16	1.78	17.54	12.44	6.04	
NAM-68	61.03	0.75	7.90	0.98	11.95	10.47	5.37	
NAM-71	54.75	1.29	6.50	1.64	14.40	12.41	6.65	
NAM-72	53.54	0.75	5.17	1.78	17.49	11.85	6.34	
NAM-73	53.05	1.23	5.37	0.67	17.71	10.55	5.88	
NAM-75	54.29	2.02	8.26	2.21	14.06	10.28	7.06	
NAM-76	51.95	0.85	6.75	2.04	17.34	11.08	7.54	
NAM-78	51.81	1.81	6.95	1.69	16.57	11.19	6.97	
NAM-79	50.21	1.09	5.48	2.27	20.83	10.70	7.13	
NAM-8	52.58	1.57	7.26	0.88	16.55	12.75	5.69	
NAM-82	60.26	1.09	4.68	1.57	13.54	10.46	6.17	
NAM-83	62.99	1.33	4.58	1.54	12.79	8.89	5.32	
NAM-85	52.70	0.86	6.84	3.60	15.53	10.88	6.37	
NAM-86	55.03	0.47	5.50	3.47	14.42	12.05	6.09	
NAM-87	51.44	1.87	6.58	3.67	14.16	11.57	7.81	
NAM-88	55.05	1.44	7.67	2.88	13.69	12.44	5.75	
YN04-C1213	52.54	2.76	8.51	0.85	13.10	12.65	6.53	
Average	53.70	1.31	6.61	2.07	15.98	11.25	6.53	
Low	42.69	0.43	4.54	0.67	11.95	8.37	5.25	
High	62.99	2.02	8.51	3.77	19.30	12.75	10.40	

4. Survey of wax diversity in *Brassica* species.

An initial survey was conducted of wax composition of other *Brassica* species within the triangle of U, *Brassica rapa, Brassica oleracea, Brassica nigra, Brassica juncea* and *Brassica carinata*. Representative varieties were chosen and plants grown in the greenhouse. Wax composition was determined from multiple tissues. The composition of leaf wax from 5 species is compared in figure 15 (data for *B. nigra* was omitted due to sample contamination). For all species alkanes were the dominant component with the C29 ketone, C29 secondary alcohol, C30 aldehyde and alkyl esters also abundant. Minor components were similar in composition. Of the 5 species *B. oleracea* leaf wax had the highest alkane levels, primarily C29 alkane, with a corresponding reduction in C29 ketone, C29 secondary alcohol and C30 aldehyde. Total alkyl esters appeared lower in *B. juncea* and this class of compounds showed significant variability (Figure 16), with higher mass components being observed in *B carinata*. A survey of wax diversity was conducted for the 50 founder varieties of the *B. carinata* NAM population. Only limited chemical diversity was observed.

Figure 15.



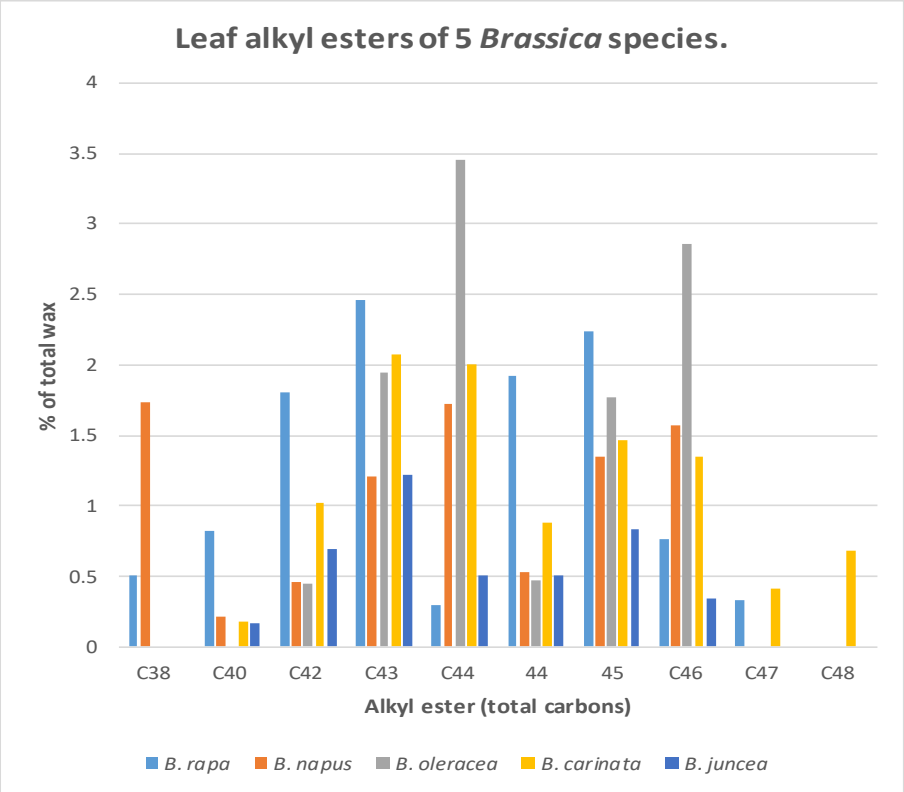


Figure 16. Comparison of alkyl ester composition for 5 *Brassica* species

5. Preliminary study of wax composition in relation to abiotic stress.

As material was available from other AAFC research activities, studies of wax composition in relation to nitrogen application and heat and drought treatment were conducted for a small number of *B. napus* varieties.

To investigate the effect of nitrogen levels on wax composition, leaves were obtained from a nitrogen use efficiency study conducted by Drs Raju Soolanayakanahally and Sally Vail at the AAFC Llewllyn farm in Saskatoon in 2019. A total of 25 lines (see table 4 for varieties studied) were surveyed with 3 replicates each for high and low nitrogen. As shown in figure 17 for varieties AC Excel and Oro, no significant variation in wax composition was observed plants grown under the high and low nitrogen treatments. The effect of more extreme nitrogen levels remains to be determined.

For the heat and drought study, leaves were obtained from greenhouse grown plants in a project conducted by Dr Raju Soolanayakanahally. Treatments were drought, heat + drought, heat + well watered and well watered. Wax was collected from leaf, pod and stem at a single time point for 4 varieties. Data from this experiment had a large amount of variation between replicates making interpretation difficult. No significant differences were seen in wax composition between treatments, but total wax load appeared increased under drought, but not heat + drought treatments (Figure 18). Measurement of

multiple growth and physiological parameters were also taken from these plants and further analysis and interpretation is in progress.

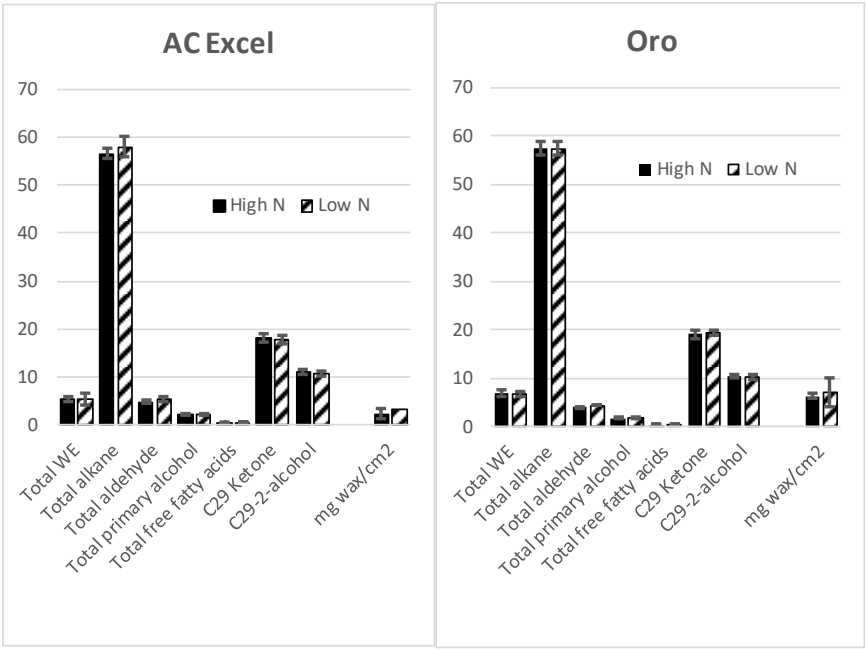


Figure 17. Wax composition of plants from varieties AC Excel and Oro grown in the field under high and low nitrogen treatments.

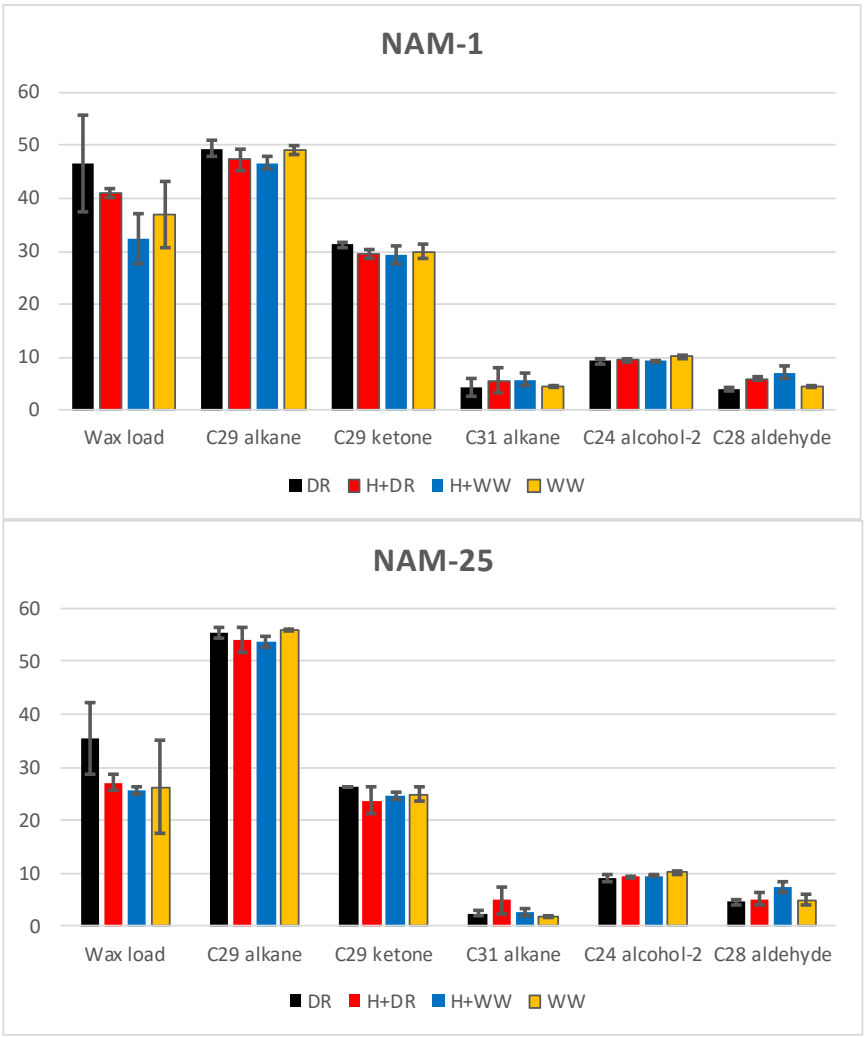


Figure 18. Wax composition of varieties NAM-1 and NAM-25 grown under heat and drought conditions. DR=drought, H+DR=heat and drought, H+WW= heat and well watered, WW=well watered. Wax composition is given as % total wax, wax load as μg/cm^2

6. Preliminary survey of NAM-RILs.

The purpose of the *Brassica napus* NAM population was to establish a population of extensively phenotypically and genetically characterized plants to act as a resource for trait characterization and manipulation. As a preliminary approach to investigate the genetic basis of differences in C30 aldehyde levels we surveyed the wax composition of 64 recombinant inbred lines (RILs) derived from a cross between NAM-0 (N99-508) and NAM-5 (BN-1). A more extensive survey was planned, but could not be conducted due to the poor growing conditions in spring 2019. Due to the very small population surveyed from a single site and single year, no significant linkage could be determined, although an unexpected correlation between (C30 aldehyde and C26 alcohol + C30 *anteiso*-alkane) was observed.

7. Developing an epidermal transcriptome to identify genes involved in wax biosynthesis.

To understand the genes involved in wax biosynthesis and their regulation an epidermal transcriptome was developed representing genes expressed in the epidermis, the tissue where wax biosynthesis occurs. To obtain material, epidermal peeling was attempted for different tissues including leaves and stems. Petiole (leaf stalk) was chosen as the tissue for which epidermis could be obtained free of underlying cell types. As epidermal cells would also be expected to expressed genes involved in cell growth and metabolism, and not involved in wax biosynthesis we also developed a transcriptome for the whole petiole, including the epidermis. By comparing the two transcriptomes, and looking for genes specifically or more highly expressed in the epidermis, genes involved in wax biosynthesis would be more likely to be identified. Three biological replicates were used for each tissue, generating 6 sets of sequence reads. A summary of reads is given in table x. Reads were mapped to the 73,996 genes identified in the B. napus DH12075 genome assembly. As shown in table 7, some reads mapped to more than 1 gene due to high sequence identity between genes. For the epidermis, reads mapped to 44,135 genes of which 37,082 were annotated with an Arabidopsis orthologue (84%). For the petiole, reads mapped to 46,801 genes of which 39261 were annotated (83%). The data was examined to identify:

1. The most highly expressed genes from each tissue
2. Genes showing differential expression with higher expression in the epidermis.
3. Expression patterns of orthologues of Arabidopsis genes known to be involved in wax biosynthesis.

Table 7. Summary of sequence reads and mapped reads for epidermal and petiole transcriptomes.

Epidermis			**Petiole**		
Library	**Total reads mapped to genes**	**Original reads**	**Library**	**Total reads mapped to genes**	**Original reads**
R1	100,631,336	72,236,716	R1P	85,246,446	49,908,962
R2	106,875,181	71,017,252	R2P	102,724,620	57,338,698
R3	116,416,065	75,612,094	R3P	97,290,645	53,582,776

Tables S1 and S2 (appendix) show the top 100 most highly expressed genes in each tissue. For epidermis, the most highly expressed genes included genes encoding proteins involved cell wall biosynthesis (30), lipid transport (8), regulatory proteins (4) and cell division and expansion (3). Fourteen genes were identified as orthologues of Arabidopsis genes of unknown function and 21 had no Arabidopsis orthologues. I gene encoded an enzyme with an Arabidopsis orthologue known to be involved in wax biosynthesis, CER1 the fatty acid decarbonylase responsible for the biosynthesis of alkanes. For the petiole samples, cell wall/cell division (13) and genes of unknown function (9) or with no Arabidopsis orthologues (27) were most highly expressed along with genes involved in cellular metabolism (10), protein turn-over (8), stress response (13) and photosynthesis (3). Genes encoding proteins involved in lipid transport and wax biosynthesis were observed in the petiole data, but not among the most highly expressed genes. A closer look at genes with no Arabidopsis orthologues showing expression in the epidermis indicates that some of the most abundant of these appear to encode putative lipid transport proteins.

A preliminary analysis of genes showing large differential analysis identified lipid transfer proteins, some proteins known to be involved in wax biosynthesis and proteins of unknown function. The identity and potential association with wax biosynthetic pathways of the unknown proteins remains to be determined.

One of the main objectives of this work was to identify *B. napus* homologues of genes known to be involved in wax biosynthesis and regulation. For this we divided the wax biosynthetic pathway, as it is currently understood in Arabidopsis (Bernard and Joubes 2013) into 13 separate activities as shown in figure 19. The Arabidopsis genes associated with each activity were identified and the *B. napus* genome was searched for potential homologous genes. For each of these the relative expression in the petiole and epidermal transcriptome data sets was determined resulting in a set of expression heat maps (Appendix Figure S1).

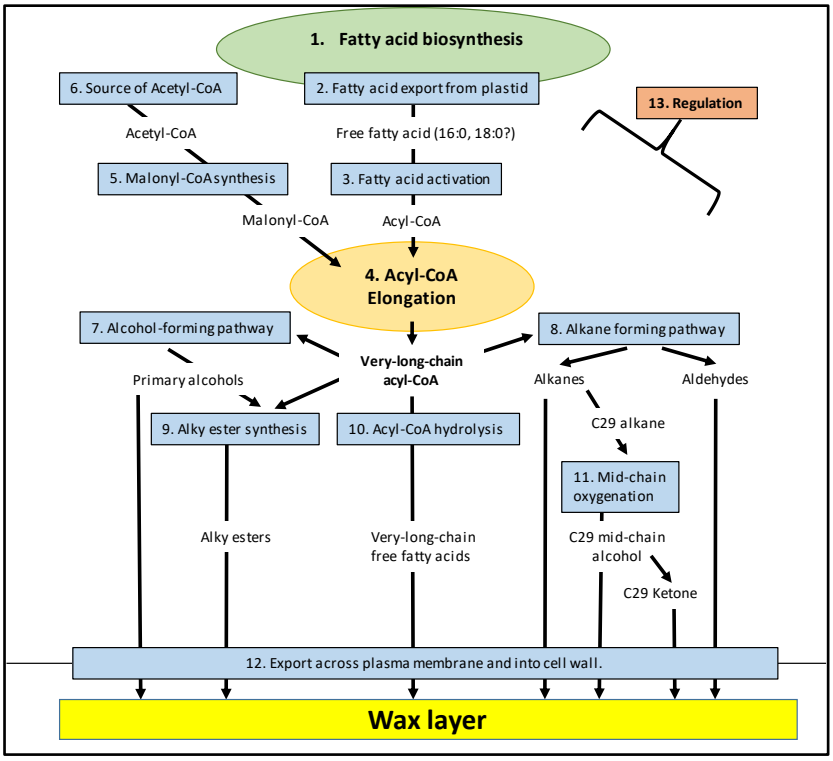


Figure 19. Diagram of the wax biosynthetic pathway in Arabidopsis. Pathway is located in the epidermal cells.

By comparing epidermal and petiole transcriptomes it was clear that certain activities, such as alkane, alcohol and alkyl ester synthesis, were controlled by genes that had high levels of expression, and which were specific to the epidermis. Perhaps surprisingly genes encoding enzymes associated with fatty acid biosynthesis, export from the plastid and the synthesis of acetyl-CoA did

not show evidence of upregulation in the epidermis, or high expression levels. The results also illustrate the utility of generating an epidermal transcriptome as a means of determining which members of multi-gene families are involved in wax biosynthesis. For example, the *B. napus* genome contains 10 genes with homology to the arabidopsis *MAH1*, the genes encoding the putative mid-chain alkane hydroxylase (MAH1) responsible for synthesis of the C29 secondary alcohol and C29 ketone (Figures 20 and S1-11).

Figure 20. Heat map showing expression patterns for candidate genes encoding MAH1 homologues. Darker green indicates higher expression.

Name	Arabidopsis gene	Arabidopsis gene ID	*B.napus* potential homologues (DH12075)	Epidermis	Whole petiole
Mid-chain alkane hydroxylase,	MAH1	At1g57750	BnaN09g16970		
			BnaN09g16960		
			BnaN09g16990		
			BnaN09g17010		
			BnaN09g17030		
			BnaN19g20540		
			BnaN19g20580		
			BnaN19g20600		
			BnaN19g20630		
			BnaN19g20640		

The petiole transcriptome data suggests that only one of these is likely to play a role in wax biosynthesis, whereas the epidermal transcriptome, through enrichment of epidermal specific mRNA reveals that 3 genes are likely to be involved. The epidermal transcriptome data indicates that the wax biosynthetic pathway is very similar to the pathway in Arabidopsis, with some exceptions. In Arabidopsis *CUT1/CER6* encodes the 3-ketoacyl-CoA synthase largely responsible for VLCFA biosynthesis in the epidermis. In *B. napus* the most highly expressed genes encoding KCS enzymes in the epidermis (Figure S1-4) are homologues of Arabidopsis CER60 and KCS1 with the CUT1/CER6 homologues appearing to play a smaller role.

The most highly expressed genes in the epidermis encode putative lipid transport proteins. These are thought to play an important, but unknown, function in wax export into the cell wall. There is also some evidence that secreted proteins may play a role in host recognition or plant defense against fungal pathogens, or may have antimicrobial properties (Pyee et al 1994, Edqvist et al 2018, Noonan et al 2017). Identifying and characterizing these proteins and their role may have implications for improvement of plant pathogen defense.

The *B. napus* genome contains a very large number of genes with potential regulatory function. Developing the epidermal transcriptome will help to narrow down the list of candidates involved in regulating aspects of wax biosynthesis. Our preliminary investigation of regulation (Figure S1-13) suggests a role for *DEWAX* and *MYB96* homologues in these processes and identified 3 out of a potential 6 candidates that could be homologues of Arabidopsis *SHINE1/WIN1* a key regulator of the whole wax biosynthetic pathway in Arabidopsis (Bernard and Joubes 2013). As our transcriptome represents a single time-point and well watered plants, the regulation of wax biosynthesis under stress remains to be determined. The different expression patterns seen among the genes encoding enzymes of wax biosynthesis suggests that regulation is complex.

Conclusions

The chemical composition of surface wax from *Brassica napus* was first reported in 1977 (Holloway et al 1977). By conducting a comprehensive analysis coupled with modern mass spectrometry tools our study was able to confirm and extend this work to identify additional low abundance components, particularly alkanes and alky esters. Unlike wheat, the chemical composition of wax in *B. napus* appears relatively uniform over the plant, with significant differences in composition only seen in petals. As petals are the primary site for sclerotinia ascospore germination (Jamaux et al 1995), further investigation into the role of petal surface chemistry and infection would be worthwhile.

Quantifying wax levels is challenging as there appears to be considerable variation in amount, even between individual leaves on a plant and identical plants within a row. If this is due to the timing of wax deposition and the environment during that time is not known. The timing and dynamics of wax deposition clearly merits further investigation. As has been observed previously, the environment has an effect on wax deposition, with increased wax evident under drought stress. We did not find evidence of a significant change in wax composition under drought conditions.

Studying wax chemical diversity with *B. napus* varieties indicates that diversity is low in this species which could limit the usefulness of natural diversity in breeding for new wax related traits. We could find no evidence of major changes in wax composition occurring during the breeding of modern canola varieties. Wax profiles differ between related *Brassica* species*, B. rapa, B. oleracea, B. juncea* and *B. carinata*, but the differences are primarily in the ratios of components. Searching for induced diversity, such as diversity in mutagenized populations may be a way to identify the germplasm needed to manipulate wax profiles if required.

By developing a transcriptome for the *B. napus* epidermis, we have been able to identify genes involved in wax biosynthesis, and potential regulatory components. As many genes in this pathway are members of multigene families, the transcriptome approach has enabled the assignment of individual genes to the pathway, and the exclusion of other very similar genes. After validation, identified genes may offer targets for manipulation, or perfect markers for breeding beneficial traits. Patterns of expression in *B. napus* reveal that the biosynthetic pathway is likely very similar to that characterized in Arabidopsis. This will be beneficial in understanding regulation and in manipulating the pathway for crop improvement. Differences in wax quantified by weight and by chemical analysis suggests that the surface wax contains other components that are likely not lipids. The high expression levels of genes encoding secreted proteins, particularly lipid transport proteins, suggests that some of the additional material may be proteins and these may have importance in biotic interactions.

The work has considerably enhanced our understanding of wax chemistry and biosynthesis in *B. napus* and identified gaps where further knowledge is required. It has established the groundwork for studies probing the role of wax in abiotic and biotic stress and provided information on the target genes that could be manipulated to modify wax to address specific challenges.

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Appendix 1.

Table S1. top 100 genes showing highest expression in *B. napus* petiole epidermis.

Rank	*Brassica napus* gene ID (DH12075)	Arabidopsis Ortholog	Arabidopsis Annotation	Process
1	BnaN17g49910	AT4G30270.1	MERI5B, MERI-5, XTH24, SEN4 | xyloglucan endotransglucosylase/hydrolase 24 | chr4:14819445-14820448	Cell wall
2	BnaN03g51590	AT4G30270.1	MERI5B, MERI-5, XTH24, SEN4 | xyloglucan endotransglucosylase/hydrolase 24 | chr4:14819445-14820448	Cell wall
3	BnaN13g16540	AT5G59310.1	LTP4 | lipid transfer protein 4 | chr5:23925296-23925772	Lipid transport
4	BnaN04g25160	AT2G38540.1	LP1, LTP1, ATLTP1 | lipid transfer protein 1 | chr2:16130418-16130893	Lipid transport
5	BnaN02g10550		No Arabidopsis Ortholog	
6	BnaN02g10540		No Arabidopsis Ortholog	
7	BnaN01g12010	AT4G21960.1	PRXR1 | Peroxidase superfamily protein | chr4:11646613-11648312	Cell wall/metabolism
8	BnaN03g38740	AT2G05520.1	GRP-3, ATGRP-3, GRP3, ATGRP3 | glycine-rich protein 3 | chr2:2026217-2026882	Cell division
9	BnaN13g48730	AT2G05520.4	GRP-3, ATGRP-3 | glycine-rich protein 3 | chr2:2026217-2026882	Cell division
10	BnaN05g06710	AT2G38530.1	LTP2, LP2, cdf3 | lipid transfer protein 2 | chr2:16128481-16128948	Lipid transport
11	BnaN03g13740	AT5G59320.1	LTP3 | lipid transfer protein 3 | chr5:23929051-23929492	Lipid transport
12	BnaN16g42310		No Arabidopsis Ortholog	
13	BnaN11g14650	AT4G21960.1	PRXR1 | Peroxidase superfamily protein | chr4:11646613-11648312	Cell wall/metabolism
14	BnaN08g19780	AT4G38770.1	PRP4, ATPRP4 | proline-rich protein 4 | chr4:18097009-18098448	Cell wall
15	BnaN06g28880	AT5G25460.1	Protein of unknown function, DUF642 | chr5:8863430-8865394	Unknown
16	BnaN12g24530		No Arabidopsis Ortholog	
17	BnaN14g07880	AT2G38530.1	LTP2, LP2, cdf3 | lipid transfer protein 2 | chr2:16128481-16128948	Lipid transport
18	BnaN12g11700		No Arabidopsis Ortholog	
19	BnaN17g36630	AT5G25460.1	Protein of unknown function, DUF642 | chr5:8863430-8865394	Unknown
20	Bna00208bs0030	AT2G34420.1	LHB1B2, LHCB1.5 | photosystem II light harvesting complex gene B1B2 | chr2:14522716-14523513	Photosynthesis

21	BnaN14g56050	AT2G38540.1	LP1, LTP1, ATLTP1 | lipid transfer protein 1 | chr2:16130418-16130893	Lipid transport
22	BnaN03g38860	AT2G06850.1	EXGT-A1, EXT, XTH4 | xyloglucan endotransglucosylase/hydrolase 4 | chr2:2763619-2765490	Cell wall
23	BnaN09g32900	AT4G11320.1	Papain family cysteine protease | chr4:6887336-6888827	Proteolysis
24	BnaN02g22600		No Arabidopsis Ortholog	
25	BnaN06g05650	AT1G09310.1	Protein of unknown function, DUF538 | chr1:3009109-3009648	Unknown
26	BnaN08g29040	AT1G09310.1	Protein of unknown function, DUF538 | chr1:3009109-3009648	Unknown
27	BnaN19g03670	AT3G29030.1	ATEXPA5, ATEXP5, ATHEXP ALPHA 1.4, EXP5, EXPA5 | expansin A5 | chr3:11011538-11013068	Cell wall
28	BnaN04g09810	AT5G38420.1	Ribulose bisphosphate carboxylase (small chain) family protein | chr5:15381203-15381978	Photosynthesis
29	BnaN12g11710		No Arabidopsis Ortholog	
30	BnaN12g34350	AT5G45280.2	Pectinacetylesterase family protein | chr5:18346862-18349488	Cell wall
31	BnaN06g29020	AT5G25610.1	RD22, ATRD22 | BURP domain-containing protein | chr5:8914498-8916684	Unknown
32	BnaN09g03410	AT3G29030.1	ATEXPA5, ATEXP5, ATHEXP ALPHA 1.4, EXP5, EXPA5 | expansin A5 | chr3:11011538-11013068	Cell wall
33	BnaN15g55650	AT3G02790.1	zinc finger (C2H2 type) family protein | chr3:604926-605243	Regulatory function
34	BnaN02g29580	AT5G45280.2	Pectinacetylesterase family protein | chr5:18346862-18349488	Cell wall
35	BnaN19g43040	AT5G57560.1	TCH4, XTH22 | Xyloglucan endotransglucosylase/hydrolase family protein | chr5:23307296-23308235	Cell wall
36	BnaN13g19240	AT2G33830.2	Dormancy/auxin associated family protein | chr2:14309768-14310286	Regulatory function
37	BnaN14g39090	AT5G38420.1	Ribulose bisphosphate carboxylase (small chain) family protein | chr5:15381203-15381978	Photosynthesis
38	BnaN11g08210	AT4G30270.1	MERI5B, MERI-5, XTH24, SEN4 | xyloglucan endotransglucosylase/hydrolase 24 | chr4:14819445-14820448	Cell wall
39	BnaN10g12470	AT5G57560.1	TCH4, XTH22 | Xyloglucan endotransglucosylase/hydrolase family protein | chr5:23307296-23308235	Cell wall
40	BnaN12g46010	AT5G25460.1	Protein of unknown function, DUF642 | chr5:8863430-8865394	Unkown
41	BnaN03g16060	AT2G33830.2	Dormancy/auxin associated family protein | chr2:14309768-14310286	Regulatory function

42	BnaN13g67710	AT4G38770.1	PRP4, ATPRP4 | proline-rich protein 4 | chr4:18097009-18098448	Cell wall
43	BnaN13g48890	AT2G06850.1	EXGT-A1, EXT, XTH4 | xyloglucan endotransglucosylase/hydrolase 4 | chr2:2763619-2765490	Cell wall
44	BnaN08g24420	AT1G20620.1	CAT3, SEN2, ATCAT3 | catalase 3 | chr1:7143142-7146193	Cell wall
45	BnaN10g03910	AT1G05850.1	POM1, ERH2, ELP1, CTL1, ELP, HOT2, ATCTL1 | Chitinase family protein | chr1:1766833-1768117	Cell wall
46	BnaN16g25180	AT1G79040.1	PSBR | photosystem II subunit R | chr1:29736085-29736781	Photosynthesis
47	BnaN03g56690	AT4G37800.1	XTH7 | xyloglucan endotransglucosylase/hydrolase 7 | chr4:17775703-17777372	Cell wall
48	BnaN12g03680	AT5G11420.1	Protein of unknown function, DUF642 | chr5:3644655-3646991	unknown
49	BnaN07g12320	AT1G20450.1	LTI29, LTI45, ERD10 | Dehydrin family protein | chr1:7088235-7089107	Stress response
50	BnaN13g52660	AT5G65730.1	XTH6 | xyloglucan endotransglucosylase/hydrolase 6 | chr5:26299080-26300290	Cell wall
51	BnaN01g32300	AT3G16640.1	TCTP | translationally controlled tumor protein | chr3:5669709-5670729	unknown
52	BnaN18g53870	AT1G02205.2	CER1 | Fatty acid hydroxylase superfamily | chr1:418818-422154	Wax biosynthesis
53	BnaN06g00740	AT1G54020.2	GDSL-like Lipase/Acylhydrolase superfamily protein | chr1:20161805-20163706	Lipid catabolism
54	BnaN12g06780	AT3G44300.1	NIT2, AtNIT2 | nitrilase 2 | chr3:15983351-15985172	Metabolism
55	BnaN02g34380	AT3G26520.1	TIP2, SITIP, GAMMA-TIP2, TIP1;2 | tonoplast intrinsic protein 2 | chr3:9722770-9723703	Vacuole function
56	BnaN13g48180	AT1G54410.1	dehydrin family protein | chr1:20310305-20310601	Stress response
57	BnaN16g26720		No Arabidopsis Ortholog	
58	BnaN18g24750	AT1G20620.1	CAT3, SEN2, ATCAT3 | catalase 3 | chr1:7143142-7146193	Cell wall
59	BnaN18g18260	AT1G09310.1	Protein of unknown function, DUF538 | chr1:3009109-3009648	Unknown
60	BnaN13g01190		No Arabidopsis Ortholog	
61	BnaN13g47650		No Arabidopsis Ortholog	
62	BnaN13g13690	AT5G56870.1	BGAL4 | beta-galactosidase 4 | chr5:23004284-23008410	Cell wall
63	BnaN06g30860	AT5G49360.1	BXL1, ATBXL1 | beta-xylosidase 1 | chr5:20012179-20016659	Cell wall
64	BnaN14g60590	AT2G45820.1	Remorin family protein | chr2:18863147-18864576	Signal transduction / pathogen interaction

65	BnaN17g36480	AT5G25610.1	RD22, ATRD22 | BURP domain-containing protein | chr5:8914498-8916684	Unknown
66	BnaN19g41170		No Arabidopsis Ortholog	
67	BnaN06g34640	AT3G25830.1	ATTPS-CIN, TPS-CIN | terpene synthase-like sequence-1,8-cineole | chr3:9447545-9450316	Secondary metabolite biosynthesis
68	BnaN02g38670	AT5G25610.1	RD22, ATRD22 | BURP domain-containing protein | chr5:8914498-8916684	Unknown
69	Bna01492s0010		No Arabidopsis Ortholog	
70	BnaN17g23130	AT5G44020.1	HAD superfamily, subfamily IIIB acid phosphatase | chr5:17712433-17714046	Cell wall
71	BnaN04g10760	AT5G39570.1	Molecular_function unknown; BEST Arabidopsis thaliana protein match is: glycine-rich protein chr5:15844021-15845827	Cell division
72	BnaN03g39390		No Arabidopsis Ortholog	
73	Bna01837s0010	AT3G02468.1	CPuORF9 | conserved peptide upstream open reading frame 9 | chr3:509815-510068	Unknown
74	BnaN09g01270	AT4G01850.2	SAM-2, MAT2, SAM2, AtSAM2 | S-adenosylmethionine synthetase 2 | chr4:796298-797479	One carbon metabolism
75	BnaN13g77400	AT4G30270.1	MERI5B, MERI-5, XTH24, SEN4 | xyloglucan endotransglucosylase/hydrolase 24 | chr4:14819445-14820448	Cell wall
76	BnaN02g28760		No Arabidopsis Ortholog	
77	BnaN03g19910		No Arabidopsis Ortholog	
78	BnaN16g44660	AT1G79040.1	PSBR | photosystem II subunit R | chr1:29736085-29736781	Photosynthesis
79	BnaN12g33050		No Arabidopsis Ortholog	
80	BnaN03g11220	AT5G56870.1	BGAL4 | beta-galactosidase 4 | chr5:23004284-23008410	Cell wall
81	BnaN02g40360	AT5G62350.1	Plant invertase/pectin methylesterase inhibitor superfamily protein | chr5:25037504-25038112	Cell wall
82	BnaN10g28580	AT5G02500.1	HSC70-1, HSP70-1, AT-HSC70-1, HSC70 | heat shock cognate protein 70-1 | chr5:554055-556334	Protein folding
83	BnaN04g25150	AT2G38540.1	LP1, LTP1, ATLTP1 | lipid transfer protein 1 | chr2:16130418-16130893	Lipid transport
84	BnaN16g43850		No Arabidopsis Ortholog	
85	Bna01837s0020	AT3G02470.4	S-adenosylmethionine decarboxylase | chr3:510223-511323	One carbon metabolism

86	BnaN13g49570		No Arabidopsis Ortholog	
87	BnaN15g32350	AT1G35720.1	ANNAT1, OXY5, ATOXY5 | annexin 1 | chr1:13225304-13226939	Stress/vesicle trafficking
88	BnaN06g25830	AT5G65730.1	XTH6 | xyloglucan endotransglucosylase/hydrolase 6 | chr5:26299080-26300290	Cell wall
89	BnaN05g22880	AT3G20820.1	Leucine-rich repeat (LRR) family protein | chr3:7280930-7282027	Cell wall
90	BnaN13g42320	AT3G16640.1	TCTP | translationally controlled tumor protein | chr3:5669709-5670729	Unknown
91	Bna05458s0010		No Arabidopsis Ortholog	
92	BnaN17g34320	AT5G49360.1	BXL1, ATBXL1 | beta-xylosidase 1 | chr5:20012179-20016659	Cell wall
93	BnaN18g13340		No Arabidopsis Ortholog	
94	BnaN12g48580	AT5G64260.1	EXL2 | EXORDIUM like 2 | chr5:25703980-25704897	Cell expansion
95	Bna06248s0010	AT1G54020.2	GDSL-like Lipase/Acylhydrolase superfamily protein | chr1:20161805-20163706	Lipid metabolism
96	BnaN06g22450	AT5G62350.1	Plant invertase/pectin methylesterase inhibitor superfamily protein | chr5:25037504-25038112	Cell wall
97	BnaN05g19150	AT1G35720.1	ANNAT1, OXY5, ATOXY5 | annexin 1 | chr1:13225304-13226939	Stess/vesicle trafficking
98	BnaN03g21430	AT2G45180.1	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein | chr2:18626377-18626781	Lipid transport
99	BnaN05g10530	AT2G33830.2	Dormancy/auxin associated family protein | chr2:14309768-14310286	Regulatory function
100	BnaN03g00890		No Arabidopsis Ortholog	

Table S2. top 100 genes showing highest expression in *B. napus* petiole.

Rank	*Brassica napus* gene ID (DH12075)	Arabidopsis Ortholog	Arabidopsis Annotation	Process
1	BnaN03g16060	AT2G33830.2	Dormancy/auxin associated family protein	Regulatory function
2	BnaN13g00850	AT5G02380.1	metallothionein 2B	Stress response
3	BnaN02g00460			Unknown
4	BnaN03g00630	AT5G02380.1	metallothionein 2B	Stress response
5	BnaN13g19240	AT2G33830.2	Dormancy/auxin associated family protein	Regulatory function
6	BnaN01g12010	AT4G21960.1	Peroxidase superfamily protein	Metabolism/stress
7	BnaN05g10530	AT2G33830.2	Dormancy/auxin associated family protein	Regulatory function
8	BnaN13g48180	AT1G54410.1	dehydrin family protein	Stress response
9	BnaN08g24420	AT1G20620.1	catalase 3	Cell wall
10	Bna00208bs0030	AT2G34420.1	photosystem II light harvesting complex gene B1B2	Photosynthesis
11	BnaN01g32300	AT3G16640.1	translationally controlled tumor protein	Unknown
12	BnaN11g14650	AT4G21960.1	Peroxidase superfamily protein	Metabolism/stress
13	BnaN19g61780	AT5G02380.1	metallothionein 2B	Stress response
14	Bna03308s0010	AT5G02380.1	metallothionein 2B	Stress response
15	BnaN09g14770			Unknown
16	BnaN14g13230	AT2G33830.2	Dormancy/auxin associated family protein	Regulatory function
17	BnaN17g49910	AT4G30270.1	xyloglucan endotransglucosylase/hydrolase 24	Cell wall
18	BnaN15g44880	AT3G15353.2	metallothionein 3	Stress response
19	BnaN07g35790	AT1G76930.2	extensin 4	Cell growth/division
20	BnaN13g42320	AT3G16640.1	translationally controlled tumor protein	Unknown
21	BnaN16g42310			Unknown
22	Bna01492s0010			Unknown
23	BnaN07g16100			Unknown
24	BnaN16g43110	AT1G76930.2	extensin 4	Cell growth/division
25	BnaN09g48720	AT2G21660.1	cold circadian rhythm and rna binding 2	Regulatory function
26	BnaN03g38440	AT1G54410.1	dehydrin family protein	Stress response
27	BnaN15g55650	AT3G02790.1	zinc finger (C2H2 type) family protein	Regulatory function
28	BnaN03g51590	AT4G30270.1	xyloglucan endotransglucosylase/hydrolase 24	Ell wall
29	BnaN18g24750	AT1G20620.1	catalase 3	Cell wall

30	BnaN07g12240	AT1G20630.1	catalase 1	Cell wall
31	BnaN19g15840	AT1G64370.1	unknown protein; Has 773 Blast hits to 375 proteins in 118 species: Archae - 0; Bacteria - 97;Metazoa - 421; Fungi - 108; Plants - 31; Viruses - 0; Other Eukaryotes - 116 (source: NCBI BLink).	Unknown
32	BnaN11g09410	AT4G29350.1	profilin 2	Cell growth/metabolism
33	BnaN19g34930			Unknown
34	BnaN14g27240	AT3G62550.1	Adenine nucleotide alpha hydrolases-like superfamily protein	Stress response
35	BnaN17g19780	AT1G20620.1	catalase 3	Cell wall
36	BnaN16g34670			Unknown
37	BnaN04g00590	AT3G62550.1	Adenine nucleotide alpha hydrolases-like superfamily protein	Stress response
38	BnaN07g35060			Unknown
39	BnaN07g28790			Unknown
40	BnaN04g09810	AT5G38420.1	Ribulose bisphosphate carboxylase (small chain) family protein	Photosynthesis
41	Bna01083s0020			Unknown
42	BnaN18g43220	AT2G21660.1	cold circadian rhythm and rna binding 2	Regulation
43	BnaN09g13460	AT1G64370.1	unknown protein	Unknown
44	BnaN09g10560			Unknown
45	BnaN01g04820	AT4G32940.1	gamma vacuolar processing enzyme	Stress response
46	Bna29670s0010	AT4G38740.1	rotamase CYP 1	Signal transduction
47	BnaN09g13050	AT1G64370.1	unknown protein; Has 773 Blast hits to 375 proteins in 118 species: Archae - 0; Bacteria - 97;Metazoa - 421; Fungi - 108; Plants - 31; Viruses - 0; Other Eukaryotes - 116 (source: NCBI BLink).	Unknown
48	BnaN19g37620	AT5G53300.4	ubiquitin-conjugating enzyme 10	Protein turn-over
49	BnaN19g15360	AT1G64370.1	unknown protein; Has 773 Blast hits to 375 proteins in 118 species: Archae - 0; Bacteria - 97;Metazoa - 421; Fungi - 108; Plants - 31; Viruses - 0; Other Eukaryotes - 116 (source: NCBI BLink).	Unknown
50	BnaN03g34490	AT3G16640.1	translationally controlled tumor protein	Unknown
51	BnaN14g43930	AT2G21660.1	cold circadian rhythm and rna binding 2	Regulation
52	BnaN01g37930	AT3G06700.3	Ribosomal L29e protein family	Metabolism
53	BnaN02g14030	AT5G53300.4	ubiquitin-conjugating enzyme 10	Protein turn-over
54	BnaN15g40660			Unknown
55	BnaN10g08190	AT5G53300.4	ubiquitin-conjugating enzyme 10	Protein turn-over

56	Bna01837s0010	AT3G02468.1	conserved peptide upstream open reading frame 9	Unknown
57	BnaN02g03330			Unknown
58	BnaN11g01080	AT4G40040.2	Histone superfamily protein	Cell division
59	BnaN03g19910			Unknown
60	BnaN11g05510	AT4G32940.1	gamma vacuolar processing enzyme	Stress response
61	BnaN01g00930	AT5G10980.1	Histone superfamily protein	Cell division
62	BnaN09g31270	AT1G28330.1	dormancy-associated protein-like 1	Regulatory function
63	BnaN18g51040			Unknown
64	BnaN13g73900			Unknown
65	BnaN01g28460	AT5G42300.1	ubiquitin-like protein 5	Protein turn-over
66	BnaN03g12250	AT5G54940.2	Translation initiation factor SUI1 family protein	Metabolism
67	BnaN13g49920	AT2G15890.1	maternal effect embryo arrest 14	Development
68	BnaN11g36670	AT5G42300.1	ubiquitin-like protein 5	Protein turn-over
69	BnaN17g34320	AT5G49360.1	beta-xylosidase 1	Cell wall/expansion
70	BnaN07g34760	AT5G44430.1	plant defensin 1.2C	Stress response
71	BnaN12g24530			Unknown
72	BnaN11g23860	AT4G16190.1	Papain family cysteine protease	Proteolysis
73	BnaN13g49940			Unknown
74	BnaN11g00450			Unknown
75	BnaN13g15560	AT5G53300.4	ubiquitin-conjugating enzyme 10	Protein turn-over
76	BnaN13g71850	AT4G19840.1	phloem protein 2-A1	Development/signalling
77	BnaN03g28250	AT3G02470.4	S-adenosylmethionine decarboxylase	One carbon metabolism
78	BnaN15g10040	AT1G13440.1	glyceraldehyde-3-phosphate dehydrogenase C2	Metabolism
79	BnaN11g13130	AT4G20260.6	plasma-membrane associated cation-binding protein 1	Metabolism
80	BnaN01g35850			Unknown
81	BnaN14g39090	AT5G38420.1	Ribulose bisphosphate carboxylase (small chain) family protein	Photosynthesis
82	BnaN13g68110	AT4G39260.3	cold circadian rhythm and RNA binding 1	Regulatory function
83	BnaN07g03810			Unknown
84	BnaN07g34780	AT5G44430.1	plant defensin 1.2C	Stress response/defense
85	BnaN10g28580	AT5G02500.1	heat shock cognate protein 70-1	Protein folding

86	BnaN13g73920			Unknown
87	BnaN09g22490			Unknown
88	BnaN12g28710			Unknown
89	BnaN04g02250			Unknown
90	BnaN14g58080			Unknown
91	BnaN08g19520			Unknown
92	BnaN09g14840	AT1G62380.1	ACC oxidase 2	Metabolism/stress response
93	BnaN03g11220	AT5G56870.1	beta-galactosidase 4	Cell wall/expansion
94	Bna01837s0020	AT3G02470.4	S-adenosylmethionine decarboxylase	One carbon matabolism
95	BnaN13g24060			Unknown
96	BnaN13g13690	AT5G56870.1	beta-galactosidase 4	Cell wall/expansion
97	BnaN02g34380	AT3G26520.1	tonoplast intrinsic protein 2	Metabolism
98	BnaN03g26450	AT1G56220.1	Dormancy/auxin associated family protein	Regulatory function
99	BnaN08g13120			Unknown
100	BnaN12g16200	AT5G53300.4	ubiquitin-conjugating enzyme 10	Protein turn-over

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Appendix 2.

Figure S1. Heat maps showing expression patterns of genes potentially involved in wax biosynthesis and its regulation in *B. napus*.

Data is shown in reads/kbp for each gene.

Colour	Reads/kbp
	No hits
	1 to 999
	1000 to 3999
	4000 to 9999
	> 10000

S1-1 Fatty acid biosynthesis

Name	Arabidopsis gene	Arabidopsis gene ID	*B.napus* potential homologues (DH12075)	Epidermis	Whole petiole
Plastidial pyruvate dehydrogenase complex	PDH-E1α	AT1G01090	BnaN18g53220		
			BnaN09g58180		
			BnaN10g00710		
			BnaN09g58090		
			BnaN15g00720		
	PDH-E1β	AT1G30120	BnaN08g20430		
			BnaN09g30130		
			BnaN15g25580		
			BnaN13g66900		
Plastid Acetyl-CoA carboxylase (Heteromeric)	CAC1-A/BCC	AT5G16390	BnaN02g06080		
			BnaN13g07840		
			BnaN03g06640		
			BnaN12g06360		
	CAC1-B/BCC	AT5G15530	Bna01346s0010		
			BnaN19g53100		
			BnaN10g19860		
			BnaN03g06200		
			BnaN19g29190		
			BnaN13g07070		
	CAC2/BC	AT5G35360	BnaN06g06400		
			BnaN15g07400		
	CAC3/Ctα	AT2G38040	BnaN13g21650		
			BnaN14g08510		
			BnaN05g07140		
			BnaN03g17980		
Malonyl CoA-ACP malonyltransferase	MCAT	AT2G30200	BnaN14g17410		
			BnaN05g13470		
			BnaN13g17200		
			BnaN04g19830		
			BnaN14g50870		
			BnaN03g14380		
Plastidial fatty acid synthase	KAS III	AT1G62640	BnaN19g17370		
			BnaN19g13920		
			BnaN09g14680		
			BnaN09g11990		
	KASI	AT5G46290	BnaN19g23790		
			BnaN17g23860		
			BnaN12g35330		
			BnaN18g13610		
			BnaN19g26800		
			BnaN09g38050		
			BnaN06g37730		
			BnaN09g19960		
			BnaN02g30260		
	ENR1	AT2G05990	BnaN07g04570		
			BnaN03g38900		
			BnaN13g48930		
			BnaN17g08200		
	KAS2/FAB1	AT1G74960	BnaN07g34110		
			BnaN02g21830		
			BnaN06g13460		
			BnaN15g15450		
			BnaN10g25400		
			BnaN16g41310		
			BnaN07g23940		
			BnaN17g45100		
			BnaN16g27460		
			Bna01167s0010		
	MTKAS	AT2G04540	BnaN13g48330		
			BnaN03g38480		

S1-2 Fatty acid export from plastid

Name	Arabidopsis gene	Arabidopsis gene ID	*B.napus* potential homologues (DH12075)	Epidermis	Whole petiole
	FATA	AT3G25110	BnaN07g05340		
FatA acyl-ACP thioesterase			BnaN17g09680		
			BnaN03g38200		
			BnaN13g47860		
			BnaN16g33480		
			BnaN04g18010		
			BnaN10g07980		
			BnaN05g27450		
			BnaN03g25850		
			BnaN16g11680		
			BnaN14g47490		
Palmitoyl-acyl carrier protein thioesterase	FATB	At1g08510	BnaN15g05910		
			BnaN09g55420		
			BnaN18g50650		
			BnaN08g29420		
			BnaN06g05110		
			BnaN18g17540		
Acyl-ACP thioesterase			BnaN04g07890		
			BnaN14g37240		

S1-3 Fatty acid activation

Colour	Reads/kbp
	No hits
	1 to 999
	1000 to 3999
	4000 to 9999
	> 10000

Name	Arabidopsis gene	Arabidopsis gene ID	*B.napus* potential homologues (DH12075)	Epidermis	Whole petiole
Long-chain acyl-CoA synthetase	LACS1/CER8	AT2G47240	BnaN13g26710.1		
			BnaN05g00590.1		
			BnaN14g61260.1		
			BnaN14g00560.1		
			BnaN14g00550.1		
			BnaN05g00580.1		
			Bna00895s0190.1		
	LACS2	AT1G49430	BnaN05g18100.1		
			BnaN15g29740.1		
	LACS3	AT1G64400	BnaN09g13100.1		
			BnaN19g15420.1		
	LACS4	AT4G23850	BnaN11g16930.1		
			BnaN01g13620.1		
	LACS6	AT3G05970	BnaN03g29650.1		
			BnaN13g36350.1		
			BnaN14g49430.1		
			BnaN05g34830.1		
			BnaN15g53060.1		
	LACS7	AT5G27600	BnaN06g30050.1		
			BnaN17g35350.1		
	LACS8	AT2G04350	BnaN10g25430.1		
			BnaN03g38390.1		
			BnaN13g48080.1		
			BnaN01g27510.1		
			BnaN16g19220.1		
			BnaN14g34160.1		
			BnaN01g21710.1		
	LACS9	AT1G77590	BnaN17g08140.1		
			BnaN07g22980.1		
			BnaN16g25990.1		
			BnaN10g22360.1		
			Bna04125s0010.1		
			BnaN13g29380.1		
			BnaN12g19050.1		
			BnaN13g26710.1		
			BnaN05g00590.1		
			BnaN14g61260.1		
			BnaN14g00560.1		
			BnaN14g00550.1		

S1-4 Acyl CoA elongation

Colour	Reads/kbp
	No hits
	1 to 999
	1000 to 3999
	4000 to 9999
	> 10000

Name	Arabidopsis gene	Arabidopsis gene ID	*B.napus* potential homologues (DH12075)	Epidermis	Whole petiole
3-ketoacyl reductase 1	KCR1	At3g11980	BnaN16g34530		
			Bna01067s0020		
			BnaN07g28630		
			BnaN02g17150		
3-hydroxyacyl-CoA dehydratase	PAS2/HCD	AT5G10480	BnaN10g23140		
			BnaN03g03880		
			BnaN13g04380		
			BnaN19g57380		
	PAS2-like	AT5G59770	BnaN12g11390		
			BnaN02g10280		
Enoyl-CoA reductase	CER10/ECR	AT3G55360	BnaN09g40190		
			BnaN18g33700		
CER2	CER2	AT4G24510	BnaN11g17730		
			BnaN01g14290		
			BnaN11g20130		
CER26	CER2-LIKE1	AT4G13840	BnaN04g07230		
			BnaN14g36610		
	CER2-LIKE2	AT3G23840	BnaN07g06360		
			BnaN17g11470		
3-ketoacyl-CoA synthase (KCS)	CUT1/CER6	AT1G68530	BnaN07g26600		
			BnaN16g31460		
			BnaN02g17900		
			BnaN12g20060		
	FDH/KCS10		BnaN04g17670		
			BnaN09g46250		
			BnaN14g48130		
			BnaN18g40470		
	HIC/KCS13	AT2G46720	BnaN05g01070		
			BnaN14g01030		
	KCS1	AT1G01120	Bna04329s0010		
			BnaN09g58200		
			BnaN10g00690		
			BnaN15g00690		
	KCS2	AT1G04220	BnaN08g31150		
			BnaN10g02290		
			BnaN15g02230		
			BnaN18g00910		
	KCS20	AT5G43760	Bna25128s0010		
			BnaN02g28720		
			BnaN02g28730		
			BnaN06g39290		
			BnaN12g32980		
			BnaN17g22830		
	KCS3	AT1G07720	Bna29734s0010		
			BnaN06g04530		
			BnaN08g29720		
			BnaN15g05200		
			BnaN18g02870		
	KCS4	AT1G19440	BnaN15g15610		
			BnaN18g44360		
	KCS5/CER60	AT1G25450	BnaN09g32730		
			BnaN15g22030		
	KCS7	AT1G71160	BnaN02g19850		
			BnaN04g15460		
			BnaN07g25510		
			BnaN16g29990		
	KCS9	AT2G16280	BnaN03g39680		
			BnaN07g03600		
			BnaN09g09870		
			BnaN13g50070		
			BnaN17g06010		
			BnaN19g11470		

S1-5 Malonyl CoA synthesis

Name	Arabidopsis gene	Arabidopsis gene ID	*B.napus* potential homologues (DH12075)	Epidermis	Whole petiole
Acetyl-CoA carboxylase (multi-domain, cytosolic)	ACC1	AT1G36160	BnaN08g06490		
			BnaN06g04330		
			BnaN16g01950		
			BnaN18g07260		

S1-6 Source of acetyl CoA

Name	Arabidopsis gene	Arabidopsis gene ID	*B.napus* potential homologues (DH12075)	Epidermis	Whole petiole
ATP citrate lyase	ACLA-1	AT1G10670	BnaN18g19390		
			BnaN08g28270		
	ACLA-2	AT1G60810	BnaN09g15620		
			BnaN19g18880		
	ACLA-3	AT1G09430	BnaN08g28940		

S1-7 Alcohol-forming pathway

Name	Arabidopsis gene	Arabidopsis gene ID	*B.napus* potential homologues (DH12075)	Epidermis	Whole petiole
Fatty acid reductase 3	CER4/FAR3	At4g33790	BnaN01g04150		
			BnaN11g04750		
			BnaN08g14100		
			BnaN13g75440		
Fatty acid reductase 2	MS2/FAR2	At3g11980	BnaN05g30720		
			BnaN11g45360		
			BnaN01g35310		
			BnaN15g48270		

Colour	Reads/kbp
	No hits
	1 to 999
	1000 to 3999
	4000 to 9999
	> 10000

S1-8 Alkane-forming pathway

Name	Arabidopsis gene	Arabidopsis gene ID	*B.napus* potential homologues (DH12075)	Epidermis	Whole petiole
VLC-aldehyde decarbonylase	CER1	AT1G02205	BnaN18g53870		
			Bna01708s0010		
			Bna25797s0010		
			Bna01708s0020		
	CER1-Like	AT2G37700	BnaN04g24680		
			BnaN14g55470		
			BnaN14g55480		
VLC-acyl-CoA reductase	CER3/WAX2	AT5G57800	BnaN03g10750		
			BnaN13g13230		
			BnaN02g11420		
			BnaN10g12700		
			BnaN03g10770		
			BnaN12g12900		
			BnaN19g43290		
Cytochrome b5	CB5-A	AT1G26340	BnaN09g33090		
			BnaN15g21700		
	CB5-B	AT2G32720	BnaN04g21600		
			BnaN05g11450		
			BnaN14g14690		
			BnaN14g53360		
			BnaN03g15500		
			BnaN13g18580		
	CB5-C	AT2G46650	BnaN04g29680		
			BnaN14g60980		
	CB5-D	AT5G48810	BnaN06g31340		
			BnaN09g04070		
			BnaN17g33850		
			BnaN19g04390		
	CB5-E	AT5G48810	BnaN02g13900		
			BnaN12g16050		
	CB5LP	AT1G60660	BnaN11g32670		
			BnaN14g23510		
			BnaN01g25610		
Cytochrome b5 reductase	CBR1	AT5G17770	BnaN19g57210		
			BnaN13g08500		
			BnaN03g07240		
			BnaN10g18220		
			BnaN02g06870		
			BnaN12g07210		

S1-9 Alkyl ester synthesis

Name	Arabidopsis gene	Arabidopsis gene ID	*B.napus* potential homologues (DH12075)	Epidermis	Whole petiole
WSD1-like O-acyl transferase	WSD1	AT5G37300	BnaN04g28050		
			BnaN05g14280		
			BnaN05g14290		
			BnaN16g14670		

S1-10 Acyl CoA hydrolysis

Name	Arabidopsis gene	Arabidopsis gene ID	*B.napus* potential homologues (DH12075)	Epidermis	Whole petiole
Acyl-CoA thioesterase		AT4G00520	BnaN13g33900		
			BnaN02g25410		
			BnaN12g27600		
			BnaN03g27510		
		AT1G01710	BnaN15g00300		
			BnaN10g00270		
Thioesterase superfamily		At5g10160	BnaN03g03760		
			BnaN13g04270		
			BnaN12g03020		
Acyl-lipid thioesterase	ALT3-like?	AT1G68260	BnaN16g34970		

S1-11 Mid-chain oxygenation

Name	Arabidopsis gene	Arabidopsis gene ID	*B.napus* potential homologues (DH12075)	Epidermis	Whole petiole
Mid-chain alkane hydroxylase,	MAH1	At1g57750	BnaN09g16970		
			BnaN09g16960		
			BnaN09g16990		
			BnaN09g17010		
			BnaN09g17030		
			BnaN19g20540		
			BnaN19g20580		
			BnaN19g20600		
			BnaN19g20630		
			BnaN19g20640		

S1-12 Export across plasma membrane and into cell wall

Name	Arabidopsis gene	Arabidopsis gene ID	*B.napus* potential homologues (DH12075)	Epidermis	Whole petiole
ABC transporter	CER5/ ABCG12	AT1G51500	BnaN16g11710		
			BnaN16g11720		
			BnaN05g17150		
	ACBG11	AT1G17840	BnaN18g23020		
			BnaN15g14180		
			BnaN08g25620		
			BnaN06g12220		
	ABCG36/ PEN3	AT1G59870	BnaN16g23780		
			BnaN07g21420		
	ABCG18	AT3G55110	BnaN18g33570		
			BnaN09g40090		
Lipid transfer protein			BnaN01g20500		
			BnaN02g10540		
			BnaN02g10550		
			BnaN03g10010		
			BnaN03g13740		
			BnaN03g41870		
			BnaN04g25150		
			BnaN04g25160		
			BnaN05g06710		
			BnaN09g37080		
			BnaN11g26010		
			BnaN12g11700		
			BnaN13g12410		
			BnaN13g16540		
			BnaN14g07880		
			BnaN14g56050		
			BnaN17g40280		
			BnaN18g30370		

S1-13 Regulation

Name	Arabidopsis gene	Arabidopsis gene ID	*B.napus* potential homologues (DH12075)	Epidermis	Whole petiole
	CER7	AT3G60500	BnaN07g20280		
			BnaN16g22030		
			BnaN18g38070		
			BnaN09g44040		
	CER9	AT4G34100	BnaN08g13920		
			BnaN13g75140		
			BnaN13g75160		
			BnaN03g54200		
			BnaN11g04570		
	CER11/CPL2	AT5G01270	BnaN03g00350		
			BnaN13g00540		
	CER16		BnaN19g22950		
			BnaN09g18840		
	DEWAX	AT5G61590	BnaN19g07370		
			BnaN17g39310		
			BnaN03g41000		
			BnaN09g06640		
	DEWAX2	AT5G07580	BnaN19g59730		
			BnaN02g02250		
			BnaN12g02040		
	SHINE1/WIN1	AT1G15360	BnaN18g21930		
			BnaN08g26410		
			BnaN15g11960		
			BnaN09g51740		
			BnaN06g10230		
			BnaN18g46610		
	GCN5	AT3G54610	BnaN04g04650		
			BnaN04g04690		
			BnaN14g33530		
			BnaN09g39580		
			BnaN14g33490		
			BnaN19g17570		
	MYB96	AT5G62470	Bna15446s0010		
			BnaN02g40410		
			BnaN19g07610		
			BnaN09g06900		
			BnaN13g56450		
			BnaN06g22560		
	SDG8	AT1G77300	BnaN16g43390		
	SDG25	AT5G42400	BnaN09g17740		
			BnaN19g21700		
	SAGL1	AT1G55270	BnaN06g00460		
			BnaN13g80610		
			BnaN16g13210		
			BnaN08g00750		
			BnaN05g15480		
			BnaN16g07700		
	WR1	AT3G54320	BnaN09g39370		
			BnaN07g17720		
			BnaN18g32750		