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Characterization of leaf cuticular waxes and cutin monomers of *Camelina sativa* and closely-related *Camelina* species

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ABSTRACT

Camelina sativa is an old world crop newly introduced to the semi-arid regions of the Southwestern US. Recently, *Camelina* gained attention as a biofuel feedstock crop due to its relatively high oil content, polyunsaturated fatty acids, very short growing season with fairly good adaption to marginal lands, and low input agricultural systems. To expand *Camelina* growing zones into more arid regions, it is important to develop new drought resistant cultivars that can grow under water-limited conditions. Plants having cuticles with low permeability to water can possess elevated dehydration avoidance and improved drought tolerance. To extend our understating of cuticle chemical composition among *Camelina* species, leaf wax and cutin monomers in seventeen accessions representing four *Camelina* species were analyzed. *Camelina* exhibited a wide range of wax and cutin contents. The primary alcohols and alkanes were the predominant classes of leaf wax, followed in abundance by wax esters, fatty acids, aldehydes, alkylguaiacols, methylalkylresorcinols, α-amyrin and ß-sitosterol. Among primary alcohols, the dominant constituents were the C_{24}, C_{26} and C_{28} homologues, while the C_{31} homologue was the most abundant alkane among all *Camelina* accessions. Cutin monomers included monohydroxy monobasic acids, phenolics, monobasic acids, monohydroxy epoxymonobasic acids, and dibasic acids. Among the cutin monomers examined, the $C_{16:0}$ diOH acid showed extensive variation among *Camelina* species.

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1. Introduction

The genus *Camelina* belongs to the Brassicaceae, a family composed of 11 known *Camelina* species (Warwick and Al-Shehbaz, 2006). Currently five species, including *C. alyssum* (Mill.) Thell., *C. hispida* Boiss, *C. microcarpa* Andrz. ex DC, *C. rumelica* Velen. and *C. sativa* (L.) Crantz are available in public plant germplasm repositories, such as The Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany (http://www.ipk-gatersleben.de/en/), U.S. National Plant Germplasm System (https://npgsweb.ars-grin.gov/gringlobal/search.aspx) and The Centre for Genetic Resources, the Netherlands (http://cgngenis.wur.nl/). Only *C. sativa* and *C. microcarpa* are currently cultivated for their oil. Commonly known as gold-of-pleasure or false flax, *Camelina* originated from Northern Europe and Southeastern Asia, recently gaining attention as a biofuel feedstock crop due to its high oil content (28–40%), polyunsaturated fatty acids (54.3%) (Budin et al., 1995; Moser and Vaughn, 2010), and very short growing season with good adaption to marginal lands and low agricultural inputs (Putnam et al., 1993; Moser and Vaughn, 2010; Obour et al., 2015). To expand *Camelina* production zones to more marginal regions, it will be important to develop new cultivars that can grow under water-limited conditions and still maintain comparably high yield and stability (French et al., 2009).

The primary mechanisms for plant adaptation to drought can be grouped into four categories that include drought escape, avoidance, tolerance and recovery (Fang and Xiong, 2015). The drought tolerance response in plants is a complex process likely triggered first by the plant's perception of dehydration in soil (or air) followed by targeted changes in metabolic responses and gene expression, and ultimately to cellular and whole plant developmental changes (Bartels and Sunkar, 2005; Golldack et al., 2014). The avoidance mechanism is generally characterized as the plant's ability to delay the onset of dehydration in its tissues as soil moisture depletes. Plants better able to avoid tissue dehydration often possess more efficient root systems that increase soil water extraction, and/or possess a higher capacity to reduce stomatal conductance, absorption of solar radiation, cuticle water permeability, and/or

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evaporative surface area (Jones et al., 1981). Non-stomatal water loss is controlled primarily by the cuticle, an extracellular, lipophilic polymer that protects the aerial organs of plants from the surrounding environment. It also provides protection from abiotic stress; for example, pathogens and insects (Jenks et al., 1994; Yeats and Rose, 2013) and stresses such as drought, heat, and supra-optimal solar radiation (Shepherd and Wynne Griffiths, 2006; Yeats and Rose, 2013; Kosma et al., 2009). The cuticle itself is composed primarily of cuticular waxes and cutin monomers, the latter composed primarily of aliphatic components cross-linked into a polyester matrix. In the model plant *Arabidopsis*, and its brassicaceous relatives, such as *Eutrema* (syn. *Thellungiella*) and *Brassica napus* L. (rapeseed), the cutin layer consists mainly of C_{16} and C_{18} ω-hydroxy and α, ω-dicarboxylic fatty acids monomers and is associated with both epi- and intra-cuticular waxes, generally dominated by derivatives of the saturated very long-chain fatty acids (VLCFA) and isoprenoids (von Wettstein-Knowles, 2001). Wax and cutin monomer composition often varies significantly between species, between organs of the same plant (Lee and Suh, 2015; Bernard and Joubès, 2013), by developmental stage (Samuels et al., 2008; Lee and Suh, 2015), and as a response to environmental conditions (Baker, 1974). The surface waxes of leaves, seeds, and roots of *Camelina sativa* cultivar 'Celine' are dominated by alkanes, primary alcohols, and free fatty acids (Razeq et al., 2014). To date, the cutin composition of *Camelina* has not been reported. With the advancement in genomics and gene discovery tools, and increased knowledge about the physiological, metabolic, and genetic determinants of important traits like the composition of the cuticle, the application of more powerful genomics-based crop improvement strategies has great potential to improve stress tolerance in crops. The objective of the current study was to extend our understanding of cuticle lipid composition and variation in *Camelina sativa* cultivars and related, non-domesticated *Camelina* species.

2. Materials and methods

2.1. Plant material and growth conditions

Seventeen accessions of four *Camelina* species, including *C. sativa*, *C. hispida*, *C. microcarpa* and *C. rumelica*, initially collected from different geographical regions, were acquired from IPK Gatersleben GenBank, Germany and/or USDA Germplasm Resources Information Network (GRIN) (Table 1). Accession 18097E was initially collected from Turkey and provided by Dr. Mark Beilstein from the University of Arizona. Two transgenic camelina lines with independent MYB96 TF insertions, C2x2.9.1 and R2x6.1.3, were also examined. C2x2.9.1 and R2x6.1.3 are improved Celine and Robinson respectively. Camelina cultivars, Celine and Robinson, were transformed with *A. tumefaciens* GV3101 strain harboring the binary construct pCAMBIA3301M1 containing *AtMyb96* gene and CaMV 35 prompter, using a transformation protocol described by Lu and Kang (2008). T1 plants were selected on one-half strength Murashige and Skoog minimal organic medium supplemented with 1% agar and mg l^{-1} glufosinolate. Multiple transgenic lines were obtained for both cultivars, and C2x2.9.1 and R2x6.1.3 were reported in this study.

Seeds were planted in 29.29 cu. in. containers of Sunshine Mix #1/LC1 (Sun Gro Horticulture, Canada), covered and vernalized at 4 °C in the dark. After five days, the containers were uncovered and moved to a growth chamber. The conditions were 12-h-light/12-h-dark at 22/20 °C and an intensity of ca. 175 μE m^{-2} s^{-1} and ambient humidity. Plants were regularly watered and fertilized with 20-20-20 fertilizer (Scotts Miracle-Grow, USA).

2.2. Leaf wax extractions and analyses

Three leaf subsamples (approximately seventh to twelfth leaf of basal rosette) from each plant (four replications) were collected at 35 days after planting (dap). Each leaf was individually submerged in 10 ml hexane (Sigma-Aldrich, USA), capped and agitated for 45 s in a 20 ml glass scintillation vial. The leaf was removed from the solvent with forceps and leaf area determined using a flatbed scanner, after which it was moved into a new vial containing 20 μl isopropanol for use in subsequent cutin analysis. The wax extracts were heated (70 °C) and reduced under N_2 until the volume could be transferred into a 2 ml glass vial. The scintillation vials were rinsed once with a few milliliters of hexane, the volume transferred again and then evaporated to dryness. For each wax sample, 90 μl of *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA, Sigma-Aldrich, USA), 10 μl ISTD (2 μg hexadecane, Sigma-Aldrich, USA) and 110 μl hexane was added for a total volume of 200 μl. The sample vials were capped and loaded onto the GC–MS.

An Agilent 7890A gas chromatograph equipped with a 5975C mass spectrometer was used for chemical identifications and quantifications. Thirty two min sample overlap was enabled to increase throughput and barcode heat each vial at 80 °C for 35 min, then mix 6 s at 2000 rpm for five cycles prior to a 1 μl splitless injection. An HP-Ultra 1 capillary column (12 m length, 200 μm inner diameter, 0.33 μm film thickness, Agilent, USA) was used, with helium as the carrier gas at 1 ml per min and temperature settings of inlet 300 °C, detector 300 °C, initial oven temperature 50 °C, then increased 20 °C per minute to 260 °C, where it was held for 8 min, then 25 °C per min to 325 °C and held again for 13.9 min for a total run time of 35 min.

For cutin analysis, the isopropanol was decanted from each vial after two days and 20 μl of $CHCl_3$: CH_3OH (1:1 v/v) with 50 mg l^{-1} butylated hydroxytoluene was added to each leaf sample. Vials were flushed with nitrogen for 1 min, capped, and stored again at −20 °C until delipidation. Before loading the soxhlets, the storage solvent was decanted and the leaf sample was transferred to liquid N_2 in a cold mortar. A pestle was used to grind the leaves, which were then rinsed into a thimble with $CHCl_3$: CH_3OH (1:1 v/v) and loaded into the soxhlets. After three days of heating, the $CHCl_3$: CH_3OH (1:1 v/v) solvent was mostly decanted, the sample poured into a culture tube and any remaining solvent removed by glass pipette. Samples were dried for three days under vacuum.

2.3. Leaf cutin depolymerization and analysis

Fifty microliters of ISTD (10 μg methyl heptadecanoate, Sigma-Aldrich, USA) was added to each delipidated sample as an internal standard. Cutin monomers were solubilized by transesterification by first adding 6 ml of 3 N methanolic HCl (Sigma-Aldrich, USA), sealing under N_2,then heating for 20 h at 60 °C. After incubation, samples were allowed to cool, caps were slowly released and 6 ml of saturated NaCl and 10 ml dichloromethane were added to each. The sample tubes were then inverted ten times and spun down at 2500 rpm for 3 min. The lower solvent phase was pipetted into a new culture tube, 5 ml of 0.9% NaCl was added, and then the samples were slightly mixed and centrifuged again. The top wash layer was pipetted off and the salt washing was done twice more for a total of three washes. The remaining lower phase was then evaporated under N_2 until ca. 500 μl remained, at which point it was transferred to a smaller 2 ml vial. One milliliter of dimethoxypropane was added to each sample, vials were capped, vortexed and incubated at 50 °C for 5 min, after which the samples were evaporated to dryness. For each cutin sample, 100 μl of *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA, Sigma-aldrich, USA), 100 μl pyridine and 200 μl heptane: toluene (1:1 v/v) was added for a total vol-

Table 1
Seventeen *Camelina* accessions representing four species from public gene banks including The Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany (http://www.ipk-gatersleben.de/en/) and U.S. National Plant Germplasm System (https://npgsweb.ars-grin.gov/gringlobal/search.aspx).

Line	Accession name	Genus species 'subsp.'	accesion name (other)	source	orgin
1	Robinson	*C. sativa* (L.) *Crantz subsp. sativa*	–	USA	USA
2	Celine	*C. sativa* (L.) *Crantz subsp. sativa*	–	France	France
3	CAM212	*C. sativa* (L.) *Crantz subsp. sativa*	STAMM 02X11A	IPK	Germany
4	CAM159	*C. sativa* (L.) *Crantz subsp. sativa*	*STAMM 13X15*	IPK	Germany
5	CAM23	*C. sativa* (L.) *Crantz subsp. pilosa*	–	IPK	–
14	PI650152	*C. sativa* (L.) *Crantz subsp. sativa*	*CPS-CAM23*	USDA	Germany
9	C2x2.9.1	*C. sativa* (L.) *Crantz subsp. sativa*		USA	USA
10	R2x6.1.3	*C. sativa* (L.) *Crantz subsp. sativa*		USA	USA
8	CAM42	*Camelina sativa* (L.) *Crantz subsp. pilosa*		IPK	USA
13	PI650143	*C. sativa* (L.) *Crantz* subsp. *sativa*	*CS-CROO*	USDA	Germany
15	PI650167	*C. sativa* (L.) *Crantz subsp. sativa*	*Index Seminum 144*	USDA	Poland
17	PI633186	*C. microcarpa Andrz. ex DC.*	*No. 61*	USDA	Hungery
6	CAM244	*C.rumelica Velen.*	Leindotter	IPK	Russia
7	18097E	*C.rumelica Velen.*		USA[a]	Turkey
11	PI650138	*C. rumelica Velen.*	*161-3724-75*	USDA	Iran
12	PI650139	*C. rumelica subsp. transcaspica*	162-3726-75	USDA	Iran
16	PI650133	*C. hispida var. grandiflora*	*158-6281-83*	USDA	Turkey, Nevsehir

[a] 18097E was collectedand donated Dr. Mark Beilstein, University of Arizona.

ume of 400 μl. The sample vials were capped and loaded onto the GC–MS.

The same GC–MS instrument was used for cutin monomer analysis, but a different method was utilized (Jenkin and Molina, 2015; Parsons et al., 2013). Eighteen minute sample overlap was enabled to increase throughput and barcode heat each vial at 80 °C for 20 min, then mix 6 s at 2000 rpm for five cycles prior to a 1 μl splitless injection. An HP-Ultra 1 capillary column (12 m length, 200 μm inner diameter, 0.33 μm film thickness, Agilent, USA) was used, with helium as the carrier gas at 1 ml per minute and temperature settings of inlet 300 °C, detector 300 °C, initial oven temperature 50 °C, then increased 15 °C per minute to 320 °C, where it was held for 2 min for a total run time of 20 min.

2.4. Interpretation of wax and cutin constituents and statistical analysis

Molecular identities of compounds were determined by characteristic quadrupole electron impact mass spectra. Many of the waxes and cutin monomers were identified by NIST, however those missing from the library were compared to previously published spectra or elucidated from their ion chromatograms. Uncorrected wax quantifications were based on total ion content and for cutin monomers, AMDIS deconvoluted target ions were utilized, each relative to the correspondingly added internal standard. Leaf surface areas were determined from scanned leaves using ImageJ (Schneider et al., 2012). Each leaf value was multiplied by two to account for both surfaces and quantified wax values are expressed as $\mu g\,dm^{-2}$. The three leaf subsamples were averaged to represent one replicate. Four replications were used for each accession.

Each constituent was analyzed using analysis of variance and the GLM procedure of SAS software, version 9.4 (Statistical Analysis System, SAS institute, 2001), to compare among accessions. Least significant differences (LSD) were calculated at significance level ($p = 0.05$).

3. Results and discussion

With the emergence of *Camelina* as a new biofuel crop for marginal, semi-arid and arid regions, a better understanding of the adaptations it might possess to help support growth in such stressful environments is needed. Increasing or otherwise modifying the *Camelina* cuticle to limit plant water loss could improve its capacity for drought avoidance by delaying the onset of tissue dehydration (Fang and Xiong, 2015; Riederer and Schreiber, 2001). In a recent report, Razeq et al. (2014) characterized leaf wax constituents of the *Camelina sativa* cultivar 'Celine'. To greatly extend these studies, we examined both leaf surface wax and cutin monomer composition and variation in 17 different accessions of *Camelina sativa* and three related *Camelina* species collected from different geographical regions worldwide. As the MYB96 transcription factor was previously used to increase leaf wax content in *Camelina* (Lee et al., 2014), two transgenic lines having independent insertions of MYB96 were also generated and examined in this study (Table 1).

3.1. Cuticular wax characterization

The *Camelina* accessions examined displayed a wide range of wax content, wherein the MYB96 transgenic line had the highest total wax ($288.10\ \mu g\,dm^{-2}$) and the Robinson variety exhibited the lowest ($72.19\ \mu g\,dm^{-2}$) (Table 2). Our results for the wax compositions of cultivar 'Celine' (Table 2) are similar to that reported by Razeq et al. (2014), however our total wax amounts were slightly lower, potentially because leaves were younger at sampling. Our results showed that *Camelina sativa* leaves produced a wax load of $137.08\ \mu g\,dm^{-2}$ when averaged across all *C. sativa* accessions (excluding the MYB96 transgenic lines). *C. rumelica* had higher total wax amount averaging $201.65\ \mu g\,dm^{-2}$ across all *C. rumelica* accessions, while *C. hispida* and *C. microcarpa* averaged $85.66\ \mu g\,dm^{-2}$ and $84.57\ \mu g\,dm^{-2}$, respectively. MYB96 is a transcription factor known to activate genes encoding very long chain fatty acid (VLCFA)-condensing enzymes involved in cuticular wax biosynthesis (Seo et al., 2011; Lee et al., 2014). Interestingly, the two MYB96 overexpressing lines produced using the USDA GRIN seed accession PI650140 exhibited leaf wax loads of 288.10 and 141.21 $\mu g\,dm^{-2}$, with the C2x2.9.1 transgenic line producing the highest wax load of all *C. sativa*, amounts significantly higher than reported for the non-transgenic parent as previously reported in Lee et al. (2014). By comparison, the non-transgenic CAM23*C. sativa* accession produced $222.15\ \mu g\,dm^{-2}$ wax load, which was actually higher than the lower MYB96 overexpressor (Table 2). It should be noted that some of the *C. sativa* accessions produced wax loads that were among the lowest of all *Camelina* examined, indicating a very broad range of wax loads present in natural accessions of *C. sativa*. The 18097E accession collected from Turkey exhibited the highest total wax amount ($226.41\ \mu g\,dm^{-2}$) among the *C. rumelica*, followed by the PI650138 accession from Iran, with $215.76\ \mu g\,dm^{-2}$ of total wax (Table 2). Besides MYB96, there are more than 20 Arabidopsis genes encoding regulatory proteins involved in wax biosynthesis (Lee and Suh, 2015; Bernard and Joubès, 2013). In the current

Table 2
Cuticular leaf wax constituents grouped by major classes for seventeen analyzed *Camelina* accessions. Three leaves were separately analyzed, then leaves subsamples were averaged to represent one replicate. Values, in μg dm^{-2}, were reported as mean of four replications, with (least squares means, α = 0.05).

Wax constituent	Chain length and Abr.	Camelina accessions																	LSD(0.05)
		C. sativa								*C. rumelica*				*C. microcarpa*				*C .hispida*	
		Robinson	Celine	CAM212	CAM159	CAM23	PI650152	C2x 2.9.1[a]	R2 x6.1.3[a]	CAM244	18097E	PI650138	PI650139	CAM42	PI650143	PI650167	PI633186	PI650133	
Primary Alcohols		**35.72**	**41.26**	**51.36**	**38.00**	**90.02**	**41.62**	**120.81**	**41.72**	**75.60**	**100.85**	**79.37**	**63.92**	**16.25**	**17.70**	**20.76**	**38.40**	**37.27**	**13.80**
Docosanol (C_{22})	C_{22} Alc	0.95	2.11	1.73	2.78	0.54	0.45	1.32	0.48	0.65	0.97	0.66	0.39	0.89	0.93	2.91	4.62	0.55	1.32
Tetracosanol (C_{24})	C_{24} Alc	17.31	19.92	16.62	15.12	19.67	11.68	31.05	11.10	10.33	19.53	14.32	5.31	3.43	5.32	7.44	13.51	14.27	5.59
Pentacosanol (C_{25})	C_{25} Alc	0.45	0.59	0.65	0.46	0.65	0.31	1.90	0.51	0.44	0.86	0.62	0.57	0.08	0.09	0.18	0.41	0.26	0.15
Hexacosanol (C_{26})	C_{26} Alc	11.30	11.84	21.74	14.09	26.85	13.49	55.05	15.26	24.24	39.69	29.43	46.85	3.55	4.65	5.31	8.72	14.62	7.46
Heptacosanol (C_{27})	C_{27} Alc	0.10	0.14	0.22	0.10	0.27	0.10	1.36	0.33	0.21	0.49	0.34	0.51	0.00	0.00	0.05	0.04	0.07	0.13
Octacosanol (C_{28})	C_{28} Alc	4.11	4.85	7.87	3.98	27.33	10.58	22.37	10.39	24.72	28.99	23.10	9.07	4.24	3.96	2.81	6.26	5.57	3.82
Nonacosanol (C_{29})	C_{29} Alc	0.06	0.09	0.12	0.05	0.41	0.13	0.61	0.22	0.39	0.33	0.31	0.06	0.00	0.00	0.04	0.00	0.11	0.07
Triacontanol (C_{30})	C_{30} Alc	1.04	1.37	1.81	1.08	12.84	4.06	4.62	2.35	11.97	6.80	7.59	0.63	2.68	1.79	0.97	3.70	1.82	1.32
Hentriacontanol (C_{31})	C_{31} Alc	0.04	0.03	0.06	0.03	0.19	0.12	0.36	0.11	0.24	0.19	0.21	0.12	0.00	0.00	0.04	0.00	0.00	0.04
Dotriacontanol (C_{32})	C_{32} Alc	0.21	0.21	0.31	0.19	0.92	0.46	1.37	0.56	1.60	1.42	1.61	0.19	0.67	0.50	0.43	0.69	0.00	0.19
Tetratriacontanol (C_{34})	C_{34} Alc	0.16	0.11	0.23	0.14	0.36	0.24	0.80	0.42	0.83	1.59	1.17	0.23	0.71	0.47	0.58	0.47	0.00	0.13
Alkanes		**15.04**	**14.03**	**23.32**	**17.14**	**71.60**	**46.47**	**101.52**	**38.79**	**71.18**	**71.57**	**81.21**	**55.80**	**35.15**	**13.42**	**12.20**	**9.07**	**37.63**	**13.36**
Pentacosane (C_{25})	C_{25} Alk	0.23	0.05	0.06	0.06	0.69	0.00	0.46	0.15	0.35	0.82	0.56	0.23	0.00	0.00	0.00	0.00	0.00	0.11
Heptacosane (C_{27})	C_{27} Alk	0.42	0.18	0.27	0.17	1.59	0.76	3.17	0.84	0.98	2.03	1.77	0.80	0.45	0.20	0.21	0.23	0.45	0.34
Nonacosane (C_{29})	C_{29} Alk	1.65	1.70	2.58	1.45	16.82	9.77	18.13	5.24	11.11	9.24	10.69	4.64	4.88	1.28	1.33	1.06	4.64	2.30
Triacontane (C_{30})	C_{30} Alk	0.37	0.16	0.28	0.18	1.09	0.42	1.42	0.42	0.63	0.51	0.49	0.48	0.55	0.21	0.21	0.39	0.21	0.18
Hentriacotane (C_{31})	C_{31} Alk	6.78	7.36	11.93	7.37	38.89	25.05	57.35	21.22	39.55	33.99	38.01	22.82	17.96	6.90	6.19	4.44	19.92	7.12
Dotriacontane (C_{32})	C_{32} Alk	0.36	0.27	0.37	0.38	0.91	0.52	1.39	0.61	0.94	1.01	1.00	0.92	0.94	0.45	0.41	0.72	0.28	0.23
Tritriacontane (C_{33})	C_{33} Alk	4.76	3.97	7.46	6.88	10.97	9.22	18.76	9.78	16.78	22.68	27.45	24.24	9.14	3.75	3.33	2.39	11.51	4.26
Pentatriacontane (C_{35})	C_{35} Alk	0.48	0.34	0.38	0.66	0.63	0.72	0.84	0.52	0.84	1.29	1.25	1.68	1.21	0.64	0.51	0.47	0.00	0.25
Wax Esters		**12.55**	**20.68**	**8.55**	**11.39**	**39.42**	**22.76**	**9.89**	**29.38**	**25.23**	**34.67**	**32.70**	**45.60**	**34.48**	**38.98**	**40.46**	**23.54**	**0.00**	**7.15**
(C_{38})	C_{38} WE	0.25	0.39	0.16	0.35	0.61	0.37	0.23	0.28	0.67	0.82	0.68	0.37	1.17	1.59	1.63	1.06	0.00	0.19
(C_{40})	C_{40} WE	2.08	2.80	1.15	1.62	6.35	2.71	1.95	2.93	3.77	4.75	4.68	3.20	4.71	6.38	5.78	3.45	0.00	0.90
(C_{42})	C_{42} WE	2.72	5.21	2.46	3.61	9.72	5.15	3.12	6.27	8.40	8.90	7.50	17.81	14.64	14.68	16.59	8.96	0.00	2.09
(C_{44})	C_{44} WE	5.91	10.04	3.75	4.64	17.30	11.03	3.24	12.51	9.33	12.42	12.09	11.18	11.38	13.39	13.29	8.01	0.00	2.90
(C_{46})	C_{46} WE	1.55	2.17	1.03	1.15	5.04	3.50	1.35	6.26	2.87	6.73	6.61	12.70	2.58	2.94	3.16	2.06	0.00	2.04
(C_{48})	C_{48} WE	0.05	0.07	0.00	0.02	0.40	0.00	0.00	1.13	0.20	1.05	1.15	0.33	0.00	0.00	0.00	0.00	0.00	0.38
Free Fatty Acids		**6.27**	**6.00**	**7.35**	**4.68**	**16.24**	**12.81**	**46.57**	**27.92**	**12.56**	**14.74**	**13.04**	**8.33**	**6.90**	**6.79**	**5.68**	**6.19**	**3.15**	**5.03**
Docosanoic acid (C_{22})	C_{22} FA	0.84	0.52	0.42	0.49	3.21	4.02	0.40	0.69	2.67	3.05	2.51	2.21	0.91	0.67	0.77	0.56	1.33	0.64
Tetracosanoic acid (C_{24})	C_{24} FA	1.03	0.81	0.52	0.61	4.22	5.04	0.83	0.95	4.54	5.38	4.07	3.81	1.55	1.06	1.20	0.95	1.67	0.74
Hexacosanoic acid (C_{26})	C_{26} FA	0.29	0.28	0.31	0.26	0.86	0.60	1.42	1.10	0.70	1.19	0.83	0.91	0.10	0.15	0.21	0.20	0.14	0.19
Octacosanoic acid (C_{28})	C_{28} FA	0.98	1.07	1.26	0.70	2.43	1.10	10.58	6.74	1.23	1.82	1.72	0.52	0.44	0.56	0.56	0.56	0.00	1.11
Nonacosanoic acid (C_{29})	C_{29} FA	0.07	0.10	0.10	0.05	0.16	0.00	1.44	0.78	0.04	0.14	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.11
Triacontanoic acid (C_{30})	C_{30} FA	1.14	1.28	1.62	0.72	3.44	1.12	13.15	6.95	1.73	1.09	1.34	0.15	1.22	1.12	0.78	1.66	0.00	1.45
Hentriacotanoic acid (C_{31})	C_{31} FA	0.08	0.09	0.14	0.07	0.09	0.00	1.38	0.71	0.04	0.08	0.22	0.00	0.00	0.00	0.02	0.00	0.00	0.09
Dotriacontanoic acid (C_{32})	C_{32} FA	1.25	1.32	1.99	1.06	1.47	0.70	12.62	6.88	1.23	1.22	1.50	0.45	1.82	2.01	1.30	1.68	0.00	1.46
Tritriacontanoic acid (C_{33})	C_{33} FA	0.04	0.05	0.09	0.05	0.00	0.00	0.66	0.34	0.00	0.00	0.02	0.00	0.00	0.00	0.01	0.00	0.00	0.06
Tetratriacontanoic acid (C_{34})	C_{34} FA	0.56	0.48	0.90	0.66	0.35	0.24	4.10	2.79	0.38	0.77	0.83	0.28	0.86	1.23	0.77	0.57	0.00	0.64
Aldehydes		**0.14**	**0.17**	**0.18**	**0.13**	**2.90**	**1.01**	**6.60**	**1.93**	**1.23**	**2.68**	**1.94**	**0.00**	**0.00**	**0.00**	**0.21**	**0.35**	**0.47**	**1.06**
Tetracosanal (C_{24})	C_{24} Ald	0.06	0.06	0.04	0.04	0.31	0.18	0.28	0.15	0.20	0.31	0.24	0.00	0.00	0.00	0.07	0.12	0.14	0.06
Hexacosanal (C_{26})	C_{26} Ald	0.03	0.03	0.06	0.04	0.49	0.27	1.00	0.32	0.24	0.81	0.48	0.00	0.00	0.00	0.04	0.05	0.13	0.18
Octacosanal (C_{28})	C_{28} Ald	0.04	0.06	0.07	0.04	1.20	0.47	3.42	1.00	0.49	1.25	0.91	0.00	0.00	0.00	0.07	0.10	0.20	0.53
Triacontanal (C_{30})	C_{30} Ald	0.01	0.02	0.01	0.01	0.73	0.09	1.90	0.46	0.31	0.22	0.23	0.00	0.00	0.00	0.03	0.05	0.00	0.32
Dotriacontanal (C_{32})	C_{32} Ald	0.00	0.00	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.09	0.08	0.00	0.00	0.00	0.00	0.02	0.00	0.06
Alkylguaiacols		**0.88**	**0.37**	**0.40**	**0.51**	**0.63**	**0.15**	**0.61**	**0.47**	**1.42**	**1.72**	**1.41**	**0.00**	**2.62**	**2.17**	**0.00**	**3.34**	**4.77**	**0.69**
(C_{19})	C_{19} AG	0.20	0.11	0.13	0.15	0.18	0.04	0.33	0.16	0.38	0.39	0.29	0.00	0.89	0.73	0.00	1.10	2.84	0.40
(C_{21})	C_{21} AG	0.61	0.24	0.25	0.33	0.37	0.11	0.28	0.31	0.85	1.12	0.94	0.00	1.47	1.18	0.00	2.24	1.93	0.31
(C_{23})	C_{23} AG	0.08	0.02	0.03	0.03	0.08	0.00	0.00	0.00	0.19	0.21	0.18	0.00	0.26	0.26	0.00	0.00	0.00	0.04
Methylalkylresorcinols		**1.21**	**0.68**	**0.47**	**0.73**	**0.39**	**0.00**	**1.54**	**0.44**	**0.22**	**0.00**	**0.00**	**0.00**	**0.01**	**0.23**	**0.00**	**3.52**	**0.41**	**0.47**
(C_{19})	C_{19} MAR	0.23	0.18	0.13	0.18	0.11	0.00	0.71	0.17	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.96	0.00	0.21
(C_{21})	C_{21} MAR	0.74	0.36	0.23	0.44	0.16	0.00	0.68	0.20	0.08	0.00	0.00	0.00	0.01	0.23	0.00	1.81	0.41	0.24
(C_{23})	C_{23} MAR	0.24	0.14	0.11	0.11	0.13	0.00	0.15	0.07	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.75	0.00	0.07
α-Amyrin	αAm	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**6.00**	**1.79**	**0.00**	**0.00**	**0.00**	**0.00**	**2.17**	**1.05**
ß-Sitosterol	BSS	**0.38**	**0.12**	**0.42**	**0.21**	**0.95**	**0.00**	**0.56**	**0.56**	**0.12**	**0.18**	**0.10**	**1.42**	**0.00**	**0.00**	**0.09**	**0.01**	**0.20**	**0.49**
Totals		72.19	83.31	92.06	72.80	222.15	124.80	288.10	141.21	187.56	226.41	215.76	176.85	95.39	79.06	79.40	84.42	86.07	27.17

[a] Two transgenic camelina lines with independent MYB96 TF insertions.

study, we reveal an extremely wide range of total wax amounts across these 17 accessions of more than 3-fold variation, indicating a likelihood that different *Camelina* species and genotypes vary significantly in the genetic regulation of their wax pathways. Recent studies have revealed the feasibility for intercrossing *C. sativa* with its related *Camelina* species (Séguin-Swartz et al., 2013), indicating that it may be possible to introgress more active cuticle genes from one species to another, and/or create new combinations of cuticle-associated genes that can increase or modify wax composition in ways to improve plant stress tolerance.

Across all *Camelina* accessions, the primary alcohols and alkanes were the predominant wax classes having 53.57 and 42.07 $\mu g\,dm^{-2}$ of average amount, respectively, trailed by wax esters, fatty acids, aldehydes, alkylguaiacols, methylalkylresorcinols, α-amyrin and β-sitostrol (Table 2, Fig. 1). *Camelina* accessions showed a wide range of primary fatty alcohols ranging from the lowest value of 16.25 $\mu g\,dm^{-2}$ for *C. microcarpa* CAM42 to the highest 100.85 $\mu g\,dm^{-2}$ for the non-transgenic *C. rumelica* 18097E, to 120.81 $\mu g\,dm^{-2}$ for the transgenic C2x2.9.1. Among the primary alcohols, the dominant constituents were the C_{24}, C_{26} and C_{28} homologues that together accounted for an average 86% of total primary alcohols across all lines. The *CER4* gene is known to encode an alcohol-forming fatty acyl-CoA reductase (FAR) responsible for the synthesis of primary alcohols in the epidermis of *Arabidopsis* aerial tissues and roots (Rowland et al., 2006) and Wang et al. (2015) reported that overexpression of the wheat *TaFAR5* gene in tomato leaves resulted in greater accumulation of C_{26}, C_{28} and C_{30} alcohols. Whether variation in the expression of *CER4* homologues in *Camelina* explains the large observed variation in primary alcohols across these *Camelina* species is uncertain. Interestingly, the C2x2.9.1*C. sativa* MYB96 overexpressor produced very high levels of primary alcohols (similar as in Lee et al., 2014), a result indicating that MYB96 may activate genes involved in the synthesis of primary alcohols.

Alkanes were the second most abundant wax class produced in all *Camelina* accessions (30.5% of total waxes, excluding the transgenic lines) with C_{29}, C_{31} and C_{33} accounting for 93% of total alkanes (Table 2). As with alcohols, the amount of alkanes on these accessions varied greatly, from as low as 9.07 $\mu g\,dm^{-2}$ for *C. microcarpa* PI633186 to as high as 81.21 $\mu g\,dm^{-2}$ for *C. rumelica* PI650138. The transgenic line C2x2.9.1 produced 101.52 $\mu g\,dm^{-2}$ of alkanes. The relative proportions of constituents within the alkane class were very similar across all accessions, except for the *C. rumelica* line PI650139, which showed a shift in the major homologue. In all other *Camelina* accessions, the most abundant alkane was the C_{31} alkane. However, in the accession PI650139, the C_{33} homologue dominated (Table 2). These results indicate a significant shift in the elongation of wax precursors, such that PI650139 is able to generate longer acyl chains feeding the alkane pathway of wax synthesis. No such genotypic variation occurs for the primary alcohol pathway among these *Camelina* accessions since no such shift in chain length distribution for primary alcohols is observed. Alkanes are considered highly hydrophobic and effective in creating cuticles with very low permeability to water, especially those having longer chain lengths (Kosma and Jenks, 2007). Increasing alkanes was observed in many species including alfalfa (Ni et al., 2012), *Populus euphratica* (Xu et al., 2016), sesame (Kim et al., 2007a) and soybean (Kim et al., 2007b) when plants were grown under drought conditions. It would be interesting to explore the *C. rumelica* line PI650139 as a parent in a breeding effort to introgress longer chain alkanes into cultivated *Camelina* as a means to reduce non-stomatal water loss.

Wax esters were the third most abundant class of waxes accounting for an average 20% of total waxes (excluding transgenic lines). *C. hispida* accession PI650133 did not produce detectable amounts of esters using our protocol, while *C. rumelica* PI650139 and *C. microcarpa* PI650167 had the highest ester deposits of 45.60 and 40.46 $\mu g\,dm^{-2}$, respectively (Table 2). The transgenic accessions did not show a major impact of the MYB96 transgene on ester profiles, except that the R2x6.1.3 line had the highest, and slightly elevated amounts of the longest C_{46} and C_{48} homologues relative to the line with the next most abundant C_{46} and C_{48} homologues of CAM23 (Table 2). Interestingly, the *C. rumelica* line PI650139 and all four of the *C. microcarpa* accessions had the C_{42} carbon homologue as their most abundant ester, rather than the C_{44} esters that dominated the other 13 *Camelina* accessions. Whether an inter-specific breeding approach might be used to modify ester chain lengths, and perhaps ester-associated cuticle permeability properties, would be an intriguing subject for future research.

The amounts of VLCFAs varied by more than 5-fold across all non-transgenic *Camelina* (Table 2), with the species *C. hispida* exhibiting very low relative amounts. Especially notable was that VLCFAs on the two transgenic lines were increased nearly 15-fold above the lowest acid line (Table 2), which was accounted for mainly by the longer, even chained C_{28}, C_{30} and C_{32} homologues. These results are comparable with the previous report by Lee et al. (2014), except under our environmental conditions the transgenic lines showed a much higher load of VLCFAs. These acid levels are in part an indicator of thioesterase activity associated with removal of the Co-enzyme-A from the acyl chain during elongation reactions, as well as the relative competition for acyl-CoA chains from other branches of the wax pathway. The very high variation among these accessions, as well as the high responsiveness to ectopic expression of MYB96, may indicate a strong potential for modifying VLCFAs using a genetic approach.

The majority of *Camelina* accessions in our panel had low or undetectable amounts of aldehydes that accounted for 1% of the total waxes ranging from 0.0–6.60 $\mu g\,dm^{-2}$ (Table 2). Several other compounds were marginally detected in the leaf surface extracts, many which have not been reported previously for *Camelina*. While α-amyrin has been detected in other Brassicaceae (Xu et al., 2014), it was only detected in three *Camelina* lines, specifically PI650138, PI650133, and PI650139, but not in others. The phytosterol ß-sitosterol was reported in *Camelina* seeds (Mansour et al., 2014), but not previously reported in leaf waxes. The accession *C. rumelica* PI650139 exhibited the highest amounts of ß-sitosterol at 1.42 $\mu g\,dm^{-2}$, followed by CAM23 that produced 0.95 $\mu g\,dm^{-2}$. A homologous series of alkylguaiacols (AGs) and methylalkylresorcinols (MARs) were detected at low levels in the leaf surface wax of saltcedar (*Tamarix canariensis* [Willd.]) (Basas-Jaumandreu et al., 2014), *Secale cereale* and *Brachypodium distachyon* (Luna, 2014), but not previously for *Camelina* or any other member of the Brassicaceae. In *Camelina*, heptadecylguaiacol (C_{17}) was detected on some *Camelina* accessions, but only in trace amounts (not shown). Many *Camelina* lines produced significant amounts of $C_{19,}$ C_{21and} C_{23} AGs, with the accession PI650133 producing the most at 4.77 $\mu g\,dm^{-2}$ (Supplemental Fig. 1), while PI633186 showed the highest amounts of MARs at 3.52 $\mu g\,dm^{-2}$, with other accessions exhibiting less than half that amount (Table 2, Supplemental Fig. 2).

3.2. Cutin monomers

Cuticular waxes and cutin monomers are the building blocks of the plant cuticle, with less being known about the synthesis of cutin, nor the role of cutin in plant stress tolerance. Cutin monomers include aliphatic $C_{16\text{-}18}$, ω-hydroxy and polyhydroxy fatty acids, and glycerol (Rautengarten et al., 2012). To date, no report of leaf cutin monomer constituents of *Camelina* has been published. Cutin constituents were extracted from *Camelina* using a chloroform/methanol-based method (Parsons et al., 2013), depolymerized, and then separated and analyzed using GC–MS and quantified by characteristic quadrupole electron impact mass spec-

Table 3
Leaf cutin monomer constituents grouped by major classes for seventeen analyzed *Camelina* accessions. Three leaves were separately analyzed, then leaves subsamples were averaged to represent one replicate. Values, in μg dm^{-2}, were reported as mean of four replications, with (least squares means, α = 0.05).

Monomer class	Chain & Abr.	*C. sativa*								*C. rumelica*				*C. microcarpa*				*C. hispida*	LSD0.05
		Robinson	Celine	CAM212	CAM159	CAM23	PI650152	C2x2.9.1	R2x6.1.3	CAM244	18097E	PI650138	PI650139	CAM42	PI650143	PI650167	PI633186	PI650133	
Alkanediols		**17.61**	**10.90**	**13.16**	**15.72**	**10.26**	**12.44**	**18.26**	**9.60**	**12.58**	**18.94**	**15.44**	**11.40**	**13.61**	**17.96**	**19.19**	**20.81**	**22.89**	**4.71**
1,18-Octadecanediol (C_{18})	$C_{18:0}$ Diol	17.42	10.56	12.78	15.47	9.95	11.95	17.78	9.26	12.09	18.52	14.89	10.65	12.82	16.86	18.63	20.50	22.52	4.67
1,22-Docosanediol (C_{22})	$C_{22:0}$ Diol	0.20	0.35	0.39	0.24	0.31	0.49	0.49	0.34	0.49	0.42	0.56	0.74	0.80	1.10	0.56	0.30	0.37	0.24
Monobasic acids		**24.16**	**23.46**	**24.63**	**21.77**	**48.84**	**57.78**	**70.35**	**56.36**	**40.43**	**46.33**	**59.42**	**110.80**	**54.51**	**57.73**	**39.88**	**47.13**	**44.54**	**17.49**
Methyl hexadecanoate (C_{16})	$C_{16:0}$ ME	9.28	8.78	10.24	7.74	19.23	31.34	24.87	26.22	19.10	18.91	28.01	46.82	24.42	23.97	14.78	19.77	25.45	5.85
Methyl octadecanoate (C_{18})	$C_{18:0}$ ME	1.31	1.90	1.46	1.28	2.97	3.13	4.36	2.95	3.17	2.54	3.15	0.00	0.00	0.00	0.00	2.37	0.00	1.29
Methyl octadecenoate (C_{18} (1))	$C_{18:1}$ ME	0.39	0.96	0.64	1.51	4.11	4.65	2.52	1.89	3.46	1.78	3.49	7.38	1.43	1.87	1.09	1.66	0.76	1.48
Methyl octadece- di & tri –enoic (C_{18} (2)) & (C_{18} (3))	$C_{18:2\&18:3}$ ME	11.29	9.99	10.01	8.94	19.75	16.36	35.51	22.38	12.07	20.37	21.14	52.96	26.48	29.48	20.61	20.03	15.43	12.36
Methyl eicosanoate (C_{20})	$C_{20:0}$ ME	0.50	0.63	0.35	0.52	0.93	0.75	0.74	0.99	0.61	0.82	0.80	0.46	1.25	0.86	1.23	1.17	0.66	0.40
Methyl docosanoate (C_{22})	$C_{22:0}$ ME	1.02	0.84	1.43	1.26	0.93	0.67	0.96	0.99	1.73	1.39	0.98	0.82	0.79	0.58	1.69	1.06	1.41	0.41
Methyl nervonate (C_{24} (1))	$C_{24:1}$ ME	0.36	0.37	0.50	0.54	0.91	0.97	0.62	0.53	1.04	0.98	1.86	2.35	0.27	0.77	0.47	0.72	1.19	0.34
Dibasic acids		**26.94**	**17.10**	**40.40**	**29.38**	**20.43**	**13.41**	**20.53**	**13.33**	**15.81**	**30.63**	**24.95**	**13.99**	**21.66**	**23.82**	**26.02**	**37.76**	**33.80**	**5.00**
Hexadecane-1,16-dioic acid (C_{16})	$C_{16:0}$ diC	12.11	6.29	17.13	10.75	9.10	7.03	6.28	5.64	5.51	12.61	10.77	6.91	12.29	12.97	10.70	14.95	13.74	2.06
Hexadecane-1,16-dioic acid (C_{16}) Me ester TMS ester	$C_{16:0}$ diC[a]	1.17	0.52	1.60	1.04	0.71	1.67	0.95	0.76	0.64	1.70	1.42	0.58	1.21	1.57	2.76	1.07	1.10	0.32
Octadecan-1,18-dioic acid (C_{18})	$C_{18:0}$ diC	0.65	0.56	0.98	0.68	0.82	0.12	0.68	0.26	0.57	0.76	0.00	0.65	0.28	0.00	1.23	0.89	1.79	0.57
Octadecene-1,18-dioic acid (C_{18} (1))	$C_{18:1}$ diC	7.03	4.31	11.11	7.89	4.62	2.30	6.74	3.40	3.87	6.00	5.49	3.70	5.96	5.91	7.55	12.22	3.68	1.50
Octadeca- di & tri –en-1,18-dioic acid (C_{18} (2)) & (C_{18} (3))	$C_{18:2\&18:3}$ diC	5.99	5.42	9.58	9.02	5.18	2.29	5.89	3.28	5.22	9.56	7.27	2.15	1.93	3.37	3.78	8.63	13.49	2.27
Monohydroxy monobasic acids		**65.71**	**37.10**	**57.47**	**59.74**	**44.99**	**48.39**	**44.02**	**32.01**	**49.80**	**64.91**	**67.79**	**48.79**	**80.14**	**81.58**	**73.24**	**70.54**	**23.24**	**10.19**
16-Hydroxy-hexadecanoic acid (C_{16})	$C_{16:0}$ ωOH	23.85	10.84	16.90	17.75	11.04	15.11	8.80	10.34	7.12	18.84	14.82	16.33	30.24	26.36	22.54	24.08	8.61	3.81
18-Hydroxy-octadecenoic acid (C_{18} (1))	$C_{18:1}$ ωOH	20.60	12.89	19.15	16.66	10.76	10.08	15.45	9.02	9.90	13.52	12.29	9.39	22.43	22.09	23.79	31.72	6.19	3.24
18-Hydroxy-octadecenoic acid (C_{18} (1)) TMS ester	$C_{18:1}$ ωOH[a]	0.61	0.28	0.38	0.26	0.50	0.72	0.51	0.36	0.18	0.26	0.22	0.11	0.98	1.40	2.96	0.68	0.00	0.42
18-Hydroxy-octadecadienoic acid (C_{18}(2))	$C_{18:2}$ ωOH	1.22	0.76	0.84	1.07	0.47	0.89	1.63	0.71	0.63	1.04	0.70	0.44	1.95	1.70	2.11	1.83	0.84	0.44
18-Hydroxy-octadecadienoic acid (C_{18}(2)) TMS ester	$C_{18:2}$ ωOH[a]	2.22	2.40	2.94	3.03	4.88	2.69	2.52	1.29	5.78	5.18	4.02	3.31	4.12	3.28	3.36	8.29	0.89	1.24
8/9/10-Hydroxy-octadecanoic acid (C_{18})	$C_{18:0}$ θOH	16.09	9.57	16.57	19.30	15.67	17.09	14.20	9.82	24.23	23.49	33.27	18.36	19.39	25.40	16.62	3.52	6.44	5.53
8/9/10-Hydroxy-octadecanoic acid (C_{18}) TMS ester	$C_{18:0}$ θOH[a]	1.13	0.37	0.70	1.68	1.67	1.81	0.92	0.46	1.97	2.58	2.47	0.85	1.03	1.36	1.87	0.44	0.28	0.62
Monohydroxy dibasic acids		**10.73**	**8.95**	**11.40**	**12.92**	**14.78**	**15.90**	**7.79**	**8.82**	**10.70**	**24.41**	**23.51**	**16.28**	**18.34**	**21.31**	**15.49**	**21.85**	**21.76**	**3.80**
7/8-Hydroxy-hexadecan-1,16-dioic acid (C_{16})	$C_{16:0}$ OH diC	10.73	8.95	11.40	12.92	14.78	15.90	7.79	8.82	10.70	24.41	23.51	16.28	18.34	21.31	15.49	21.85	21.76	3.80
Dihydroxy monobasic acids		**81.69**	**59.87**	**106.97**	**74.20**	**88.61**	**150.54**	**87.64**	**54.45**	**49.08**	**170.89**	**139.76**	**111.88**	**240.05**	**224.29**	**203.08**	**211.47**	**72.13**	**39.29**
8/9/10, 16-Dihydroxy-hexadecanoic acid (C_{16})	$C_{16:0}$ diOH	72.71	52.45	95.72	66.21	71.58	129.43	78.55	50.39	37.09	151.43	128.83	99.14	220.28	203.98	173.83	187.95	64.06	36.85
8/9/10, 16-Dihydroxy-hexadecanoic acid (C_{16}) TMS ester	$C_{16:0}$ diOH[a]	6.28	3.67	7.70	4.97	9.67	13.05	5.71	2.05	6.33	12.02	6.60	6.60	9.17	10.54	15.66	10.09	1.80	2.65
9/10/11, 18-Dihydroxy-octadecanoic acid (C_{18}) *erythro*	$C_{18:0}$ diOH	2.20	3.26	3.05	2.66	5.58	6.11	2.59	1.56	4.91	6.24	3.85	5.41	9.07	6.55	10.28	9.12	5.60	1.45
9/10/11, 18-Dihydroxy-octadecenoic acid (C18($_1$))	$C_{18:1}$ diOH	0.50	0.49	0.50	0.37	1.78	0.99	0.80	0.46	0.76	1.21	0.49	0.72	1.53	1.00	2.02	3.05	2.33	0.66
Dihydroxy dibasic acids		**7.63**	**4.77**	**3.77**	**8.43**	**7.09**	**1.64**	**10.28**	**3.45**	**5.08**	**2.77**	**6.18**	**1.34**	**6.73**	**5.73**	**4.48**	**0.54**	**0.39**	**3.35**
9, 10-Dihydroxyoctadecane-1,18-dioic acid (C_{18}) *erythro*	$C_{18:0}$ diOH diC *e*	3.10	1.12	0.00	3.15	7.09	7.10	5.36	1.66	4.10	1.44	2.23	0.00	2.95	3.44	2.06	2.90	2.99	3.66
9, 10-Dihydroxyoctadecane-1,18-dioic acid (C_{18}) *threo*	$C_{18:0}$ diOH diC *t*	4.53	3.66	3.77	5.28	0.00	1.64	4.92	1.79	0.97	1.33	3.95	1.34	3.78	5.73	4.48	0.54	0.39	1.45
Trihydroxy monobasic acids		**4.55**	**5.23**	**3.32**	**6.11**	**13.21**	**17.44**	**18.61**	**5.15**	**12.92**	**30.45**	**20.11**	**14.27**	**10.94**	**14.98**	**9.19**	**11.47**	**17.61**	**8.22**
9, 10, 16-Trihydroxyoctadecenoic acid (C_{16})	$C_{16:0}$ triOH	1.11	0.48	0.46	1.43	1.13	1.34	0.20	0.09	0.88	2.16	1.34	1.67	2.00	2.22	1.85	2.64	0.27	0.48
9, 10, 18-Trihydroxyoctadecenoic acid (C_{18})	$C_{18:0}$ triOH	1.34	2.02	1.17	1.42	1.92	6.19	9.29	2.93	3.07	10.89	3.38	3.13	5.63	5.11	3.19	0.82	4.79	3.71
9, 10, 18-Trihydroxyoctadecenoic acid (C_{18}(1))	$C_{18:1}$ triOH	0.08	0.16	0.00	0.16	1.19	1.84	1.00	0.25	1.29	2.88	1.81	0.71	0.45	0.85	0.22	0.84	0.21	1.01
9, 10, 18-Trihydroxyoctadecadienoic acid (C_{18}(2))	$C_{18:2}$ triOH	2.03	2.57	1.70	3.11	8.98	8.08	8.12	1.89	7.68	14.52	13.59	8.76	2.86	6.80	3.92	7.18	12.34	5.04
Monohydroxy epoxymonobasic acids		**21.68**	**18.65**	**20.29**	**23.11**	**22.20**	**21.09**	**28.14**	**7.89**	**17.13**	**14.03**	**23.83**	**4.66**	**12.98**	**19.39**	**13.23**	**9.31**	**17.00**	**5.48**
9, 10-Epoxy-18-hydroxyoctadecanoic acid (C_{18})	$C_{18:0}$ Ep ωOH	8.77	6.61	9.27	9.04	9.90	7.89	9.02	2.16	6.90	3.54	8.98	1.75	6.86	9.26	5.82	4.98	1.05	2.33
9, 10-Epoxy-18-hydroxyoctadecenoic acid (C_{18}(1))	$C_{18:1}$ Ep ωOH	1.50	1.22	1.35	1.80	1.85	1.71	2.18	0.42	1.28	1.02	1.25	0.00	1.26	1.22	1.23	0.25	1.60	1.03
9, 10-Epoxy-18-hydroxyoctadecadienoic acid (C_{18}(2))	$C_{18:2}$ Ep ωOH	11.41	10.82	9.67	12.27	10.45	11.48	16.94	5.31	8.96	9.47	13.60	2.91	4.87	8.93	6.19	4.08	14.35	3.06
Phenolics		**38.41**	**25.72**	**34.64**	**38.40**	**42.26**	**54.65**	**49.33**	**14.72**	**49.77**	**66.71**	**25.10**	**31.02**	**51.82**	**43.00**	**46.75**	**116.55**	**79.26**	**21.95**
Coumaric acid	pCA	5.63	4.04	6.94	7.39	20.64	29.15	5.55	1.09	12.40	40.78	17.55	7.35	30.23	25.26	23.83	71.96	72.45	16.82
Ferulic acid	FA	2.36	3.01	1.85	3.25	4.36	2.99	3.54	2.35	1.20	1.93	0.81	1.59	7.39	4.68	6.51	15.46	2.95	2.18
Caffeic acid	CA	28.20	17.66	22.93	26.34	13.11	20.79	37.28	9.50	34.67	20.26	5.79	17.66	6.67	6.31	7.91	14.72	0.14	10.06
Sinapinic acid	SA	2.22	1.02	2.94	1.42	4.16	1.72	2.96	1.78	1.50	3.75	0.96	4.43	7.52	6.75	8.50	14.42	3.73	2.80
Total Cutin		299.12	211.75	316.04	289.76	312.65	393.26	354.93	205.78	263.29	470.06	406.09	364.41	510.77	509.80	450.54	547.43	332.61	78.42

[a] Denote a TMSi or diTMSi ester.

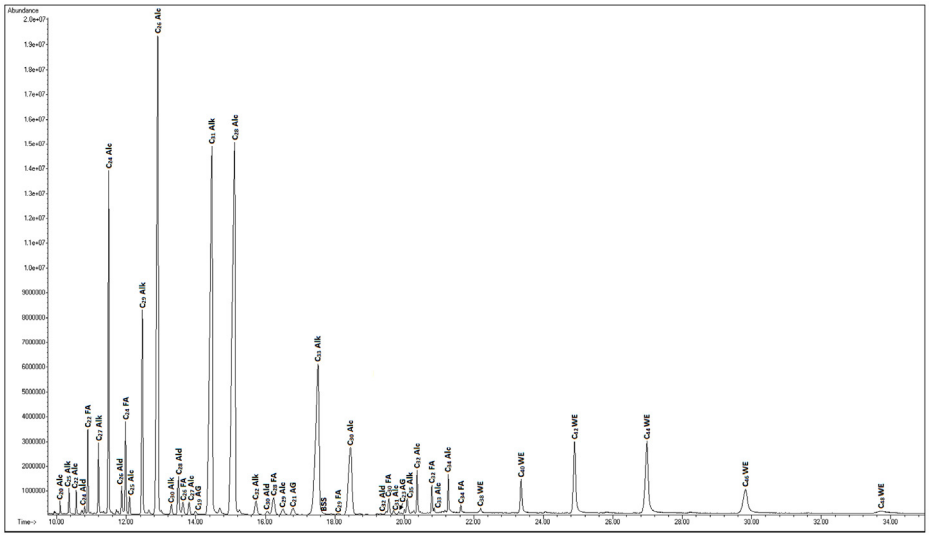

Fig. 1. Total ion chromatogram of retention times 10.0–35.0 min for *Camelina rumelica* cuticular leaf wax. C_n denotes chain length with abbreviations as listed: AG = alkylguaiacol, Alc = fatty alcohol, Ald = aldehyde, Alk = alkane, BSS = ß-Sitosterol, FA = free fatty acid and WE = wax ester. Of the seventeen analyzed accessions, α-amyrin was detected in three and methylalkylresorcinols in thirteen (these compounds are not depicted as they were not present in this accession).

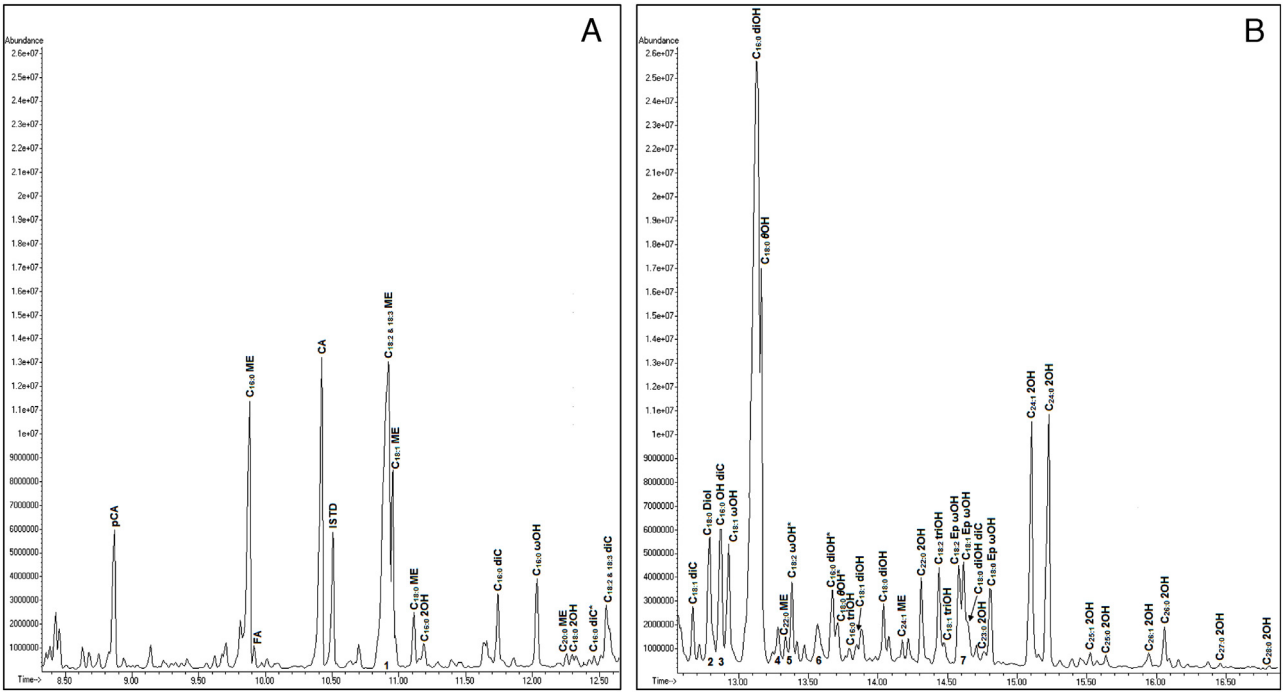

Fig. 2. Total ion chromatogram for *Camelina rumelica* leaf cutin. (A) Retention times 8.4–12.6 min, constituents numbered within the peaks at baseline are minor coelution compounds extracted with AMDIS. 1 = SA. *Asterisks denote a TMSi or diTMSi ester. (B) Retention times 12.6–16.8 min, constituents numbered within the peaks at baseline are minor coelution compounds extracted with AMDIS. 2 = $C_{18:2}$ ωOH, 3 = $C_{18:0}$ diC, 4 = $C_{22:0}$ Diol, 5 = $C_{20:0}$ 2OH, 6 = $C_{18:1}$ ωOH* and 7 = $C_{18:0}$ triOH. *Asterisks denote a TMSi or diTMSi ester.

tra (Jenkin and Molina 2015). Since overexpression of MYB96 did not have a notable effect on accumulation of cutin monomers in these accessions (Table 3), it appears that MYB96 has its primary effect on wax and not cutin pathways. Fig. 2, is a representative (*C. rumelica*) GC–MS total ion chromatogram of depolymerized *Camelina* leaf cutin. *Camelina* species showed significant variation in cutin monomer content, wherein total cutin monomers in *C. microcarpa* were the highest, averaging 504.64 μg dm^{-2}, followed by *C. rumelica* at 375.96 μg dm^{-2}, *C. hispida* at 332.61 μg dm^{-2}, and then *C. sativa* at 297.91 μg dm^{-2} (Table 3). For individual accessions, *C. microcarpa* PI633186 displayed the highest total amount of cutin monomers at 547.43 μg dm^{-2}, and this was mainly attributable to elevated phenolics and dihydroxy monobasic acids. The lowest of the seventeen accessions was *C. sativa* 'Celine' that produced 211.75 μg dm^{-2} of total cutin monomers. Within each species, cutin constituents varied greatly as well. For example, total cutin monomer amount on *C. sativa* accessions ranged from 211.75 μg dm^{-2} to 393.29 μg dm^{-2}. The main *Camelina* cutin monomers identified included the monohydroxy monobasic acids, phenolics, monobasic acids, monohydroxy epoxymonobasic acids and dibasic acids, and low abundance constituents included alkanediols, monohydroxy dibasic acids, dihydroxy dibasic acids, and

trihydroxy monobasic acids (Supplemental Figs. 3–6). The dihydroxy monobasic acids group contains $C_{16:0}$ diOH, $C_{18:0}$ diOH and $C_{18:1}$ diOH acids, which accounted for 34% of total cutin monomers (Table 3). Unlike *Arabidopsis* leaves, where the major monomer is octadeca-*cis*-6, *cis*-9-diene-1, $C_{18:2}$ diOH acid (Bonaventure et al., 2004), the C_{16} carbon monomer, 16-dihydroxy-hexadecanoic acid ($C_{16:0}$ diOH acid) was most abundant for *Camelina* (Table 3). The $C_{16:0}$ diOH acid accounted for 30% of the total *Camelina* cutin monomers. *Camelina* accessions had large variation in the amount of $C_{16:0}$ diOH acids, ranging from 37.09 $\mu g\,dm^{-2}$ in CAM244 to 220.28 $\mu g\,dm^{-2}$ in CAM42 (40% of total cutin in CAM42). The $C_{16:0}$ diOH acid was the major cutin monomer in canola (Bonaventure et al., 2004) and cucumber (Gérard et al., 1994) leaves. Xu et al. (2016) demonstrated that the major cutin monomers in leaves of *Populus euphratica* were 10,16-diOH $C_{16:0}$ acids, that were 1.5 fold more abundant on leaves growing in a more arid than less arid location. These findings suggest that *Camelina*'s most abundant $C_{16:0}$ diOH monomer could contribute to *Camelina*'s reported stress tolerance, and its levels among diverse genotypes could serve as a biomarker for drought stress tolerance in *Camelina*. Future cutin characterization studies are warranted.

Phenolic acids ranged from 14.72 to 116.55 $\mu g\,dm^{-2}$ and accounted for 13% of total cutin monomers (Table 3). Coumaric and caffeic acids contributed 47% and 36% of total phenolics. There tended to be an inverse relationship between coumaric and caffeic acids between *C. sativa* and *C. microphylla* ($r = -0.47107$; $Prob > 0.0563$), wherein an accession that exhibited high caffeic amount would exhibit low coumaric acid amount, and vice versa (Table 3). Whether this is influenced by shunting within related metabolic pathways is unknown. Ferulic and sinapinic acids accounted together for 16.6% of the total phenolics, wherein *C. microcarpa* PI633186 exhibited the highest for both (at 15.46 and 14.42 $\mu g\,dm^{-2}$, respectively), and also had highest total phenolic acid class production (116.55 $\mu g\,dm^{-2}$) (Table 3).

The monobasic acids were represented by a series including $C_{16:0}$ to $C_{24:1}$ acids (as methylated depolymerization products) that accounted for 13% of the total *Camelina* cutin monomers, whose totals ranged from 21.77–110.80 $\mu g\,dm^{-2}$ among different accessions. The $C_{16:0}$ and $C_{18:2\&18:3}$ fatty acids were the most abundant in this group, with *C. sativa* accession CAM159 exhibiting the lowest $C_{16:0}$ and $C_{18:2\&18:3}$ acids content of 7.74 $\mu g\,dm^{-2}$ and 8.94 $\mu g\,dm^{-2}$, respectively, and *C. rumelica* PI650139 having the highest amounts of those acids (Table 3).

The hydroxy acids consisted of C_{16} and C_{18} families, and accounted for 15% of the *Camelina* cutin monomers (Table 3). The *Camelina* accessions varied from 23.24 to 81.58 $\mu g\,dm^{-2}$. Among these acids, $C_{16:0}$ ω OH, $C_{18:1}$ ω OH and $C_{18:0}$ θ OH acid had higher levels with around 88% of α, θ-monohydroxy monobasic acids.

Monohydroxy epoxy acids constituted 4% of total *Camelina* cutin monomer amount. The composition of these acids varied among *Camelina* accessions, for example 9,10-Epoxy-18-hydroxyoctadecadienoic acid ($C_{18:2}$) contributed to 60% (16.94 $\mu g\,dm^{-2}$) in the transgenic C2x2.9.1 (28.14 $\mu g\,dm^{-2}$ total) (Supplemental Fig. 6, Table 3), while it attributed 85% (14.35 $\mu g\,dm^{-2}$) of monohydroxy epoxy acids produced in *C. hispida* PI650133 (17.00 $\mu g\,dm^{-2}$ total). Accession *C. rumelica* 18097E displayed the highest total trihydroxy monobasic acid (Supplemental Fig. 5) with an amount of 30.45 $\mu g\,dm^{-2}$, and *C. sativa* CAM212 produced the lowest amount (Table 3). Across accessions, $C_{18:2}$ triOH followed by $C_{18:0}$ triOH acids composed most of the trihydroxy monobasic acids (Table 3).

3. Conclusions

Under drought stress conditions, plants are generally adapted to employ more than one mechanism to resist drought, and these could involve a combination of different traits including those that are morphological, physiological, and/or biochemical. Cuticle composition is considered part of a drought avoidance mechanism that delays the onset of dehydration as water becomes increasingly limiting. The current study characterized leaf cuticular wax and cutin monomers of 17 *Camelina* accessions representing four different species. Wide variations in total wax and cutin amount and chemical constituent profile within and among *Camelina* species were observed. Major wax and cutin constituents, such as primary alcohols, alkanes, and dihydroxy acids identify potential targets for breeding efforts to improve drought tolerance in *Camelina*, potentially by the creation of interspecific hybrids or transgenic approaches. Future studies to elucidate *Camelina* cuticle metabolic pathways, the ecological function of specific cuticle lipid profiles, and the genetic networks regulating their expression, are needed to lay the groundwork for future application of new genomics-based strategies to improve environmental stress tolerance in *Camelina*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2017.01.030.

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