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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-