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Characterization of leaf cuticular wax classes and constituents in a spring *Camelina sativa* diversity panel

Pernell Tomasi[a], John M. Dyer[a], Mathew A. Jenks[b], Hussein Abdel-Haleem[a,*]

[a] USDA-ARS, US Arid-Land Agricultural Research Center, 21881 North Cardon Lane, Maricopa, AZ 85138, USA
[b] Division of Plant and Soil Sciences, West Virginia University, Morgantown, WV, 26505, USA

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ABSTRACT

Among oilseed species, Camelina has received considerable attention as an oilseed crop that can be manipulated easily to meet important non-food bioenergy requirements, where it is relatively high in oil content and polyunsaturated fatty acids, and has a very short growing season with fairly good adaption to marginal lands and low input agricultural systems. To expand Camelina cultivation zones into more arid regions, it is important to develop new drought resistant cultivars that can grow under water-limited conditions. Increasing accumulated leaf cuticular wax in Camelina could be one of the strategies to reduce nonstomatal water loss and thus increase crop tolerance to drought. To extend the understanding of phenotypic variations in cuticular wax content and composition in *Camelina sativa*, leaf wax constituents from a spring Camelina diversity panel containing 163 accessions were extracted and analyzed. The diversity panel exhibited a wide range in total leaf wax contents, wax classes and constituents. 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. High heritability values of the primary alcohol class and its dominant constituent C_{24}, C_{26} and C_{28} homologues, as well as the alkane class and abundant C_{29}, C_{31}, and C_{33} constituents, suggested the feasibility for selection of these traits during early generations of Camelina breeding programs. Positive correlations among leaf wax content, wax classes and their constituents suggest that modifying specific wax constituents could increase the wax loads, which in turn could enhance cuticle composition and properties. Quantification of leaf wax traits in the Camelina diversity panel will underpin future analysis of the Camelina wax biosynthetic pathways, help dissect its genetic regulatory elements, identify candidate genes controlling these traits, and enable the development of molecular markers for molecular breeding programs aimed at increasing drought tolerance of Camelina.

1. Introduction

There is a growing need to develop high-yielding non-food oil crops that can be cultivated in marginal farming areas for biodiesel uses. The ideal biodiesel crop should have high oil content, favorable fatty acid composition, compatibility with existing farm equipment and infrastructure, marginal growth conditions including mild water deficit, have definable growing seasons, and uniform seed maturation rates (Moser and Vaughn, 2010). Among oilseed species such as canola, soybean, rapeseed and sunflower, *Camelina sativa* has received considerable recent attention as an oilseed crop that can be manipulated easily for desired lipid compositions and meet important non-food bioenergy and bioproducts end uses (Iskandarov et al., 2014). Camelina is an old world crop newly introduced to the semi-arid west of the U.S. Even though Camelina is lower yielding than canola (a food oil crop), it has shorter life cycles that have placed it as a potential candidate for spring-sown crop rotations. Camelina also grows fairly well in marginal lands with low inputs compared to other oilseed crops, and has high seed oil content (28–40%) compared to soybean (18–22%) (Budin et al., 1995). Camelina oil has a relatively high content of polyunsaturated fatty acids (54.3%), but the methyl esters derived from the oil are compatible, both neat and blended, with petro-diesel (Moser et al., 2016; Moser and Vaughn, 2010). Camelina has well-developed genomics resources include a reference genome sequence (Abdullah et al., 2016; Kagale et al., 2014; Kagale et al., 2016). These resources will be useful for developing novel traits through molecular breeding, genome editing, and other genetic engineering approaches as well as identification of genes and markers underpinning natural variation within important agronomic traits.

Reduction of cuticle water permeability is one of the drought avoidance mechanisms that plant species evolved to tolerate water-limited growing conditions (Jones et al., 1981). Cuticlar wax

* Corresponding author.
E-mail address: Hussein.abdel-haleem@ARS.USDA.GOV (H. Abdel-Haleem).

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compositions often vary significantly between species, between organs of the same plant (Bernard and Joubès, 2013; Lee and Suh, 2015; Razeq et al., 2014; Samuels et al., 2008), and in response to abiotic and biotic stresses (Kosma et al., 2009; Kosma and Jenks, 2007; Shepherd and Griffiths, 2006; Xue et al., 2017; Yeats and Rose, 2013). The major plant leaf wax classes are free fatty acids, primary alcohols, alkanes and aldehydes. It was reported that leaf wax content and constitutes increased in response to abiotic stress (Xue et al., 2017), specifically to drought stress in plant species such as Arabidopsis (Kosma and Jenks, 2007), alfalfa (Ni et al., 2012), *Populus euphratica* (Xu et al., 2016), sesame (Kim et al., 2007a), soybean (Kim et al., 2007b), tobacco (Cameron et al., 2006) and maritime pine (Le Provost et al., 2013).

Tomasi et al. (2017) found that Camelina species exhibited a wide range of wax contents, with primary alcohols and alkanes as the predominant classes of leaf wax, followed in abundance by wax esters, fatty acids and aldehydes. 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 species (Tomasi et al., 2017).

The goal of current research is to extend the understanding to the phenotypic variations in leaf wax traits within domesticated *Camelina sativa* diversity panel. Leaf waxes of a spring Camelina diversity panel consisting of accessions typically grown in different geographical regions were extracted and analyzed. The specific objectives of the current study were to detect and characterize the phenotypic variation in *Camelina sativa* leaf wax, and estimate the genetic components and heritability estimates of these wax traits. The he longer-term goal is to identify candidate genes controlling wax biosynthetic pathways for improving Camelina drought tolerance using genomics-based crop improvement strategies.

2. Materials and methods

2.1. Plant materials

A Camelina diversity panel consisting of 163 accessions of *Camelina sativa* was obtained from Plant Gene Resources of Canada (PGRC, Saskatoon, SK, Canada; http://pgrc3.agr.gc.ca). The panel represents spring Camelina accessions collected from different regions of Europe (Supplementary Table 1). The accessions were planted under greenhouse conditions at the US Arid-Land Agricultural Research Center in Maricopa, Arizona during March 2016. The accessions were arranged in randomized complete block design (RCBD) with three replications each. The seeds of each accession were planted in 29.29 cu. in. containers of Sunshine Mix #1/LC1 (Sun Gro Horticulture, Canada). Plants were regularly watered and fertilized with N-P-K 20-20-20 fertilizer (Scotts Miracle-Grow, USA).

2.2. Wax extraction and analyses

Wax extraction and analyses followed the Camelina leaf wax extraction protocols described in Tomasi et al. (2017), with slight modifications. Briefly, sample consists of three leaves (approximately seventh to twelfth leaf from basal rosette) from each replicate was collected at 35 days after planting. Immediately, leaves were submerged in 10 mL hexane (Sigma-Aldrich, USA) in a 20 mL glass scintillation vial, then three internal standards were added including: 10 μg nonadecanoic acid, 10 μg tetracosane and 20 μg tricosanol. Vials were capped and agitated for 45 s. The leaves were removed from the solvent with forceps and leaf area was determined using a flatbed scanner and ImageJ (Schneider et al., 2012). The wax extracts were heated (70 °C) and reduced under N_2 until the volume could be transferred to a 2 mL glass vial. The scintillation vials were rinsed once with a few mL of hexane, the volume transferred again and then evaporated to dryness. For each sample, 100 μL of *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA, Sigma-Aldrich, USA) and 100 μL hexane was added for a total volume of 200 μL. The sample vials were capped and loaded onto the Agilent 7890A gas chromatograph equipped with a 5975C mass spectrometer. Each vial was heated 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 a 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.

2.3. Interpretation of wax constituents and statistical analysis

Molecular identities of compounds were determined by characteristic quadrupole electron impact mass spectra method, as described by Tomasi et al. (2017). Uncorrected wax quantifications were based on specific target ions relative to the corresponding added internal standard. Leaf surface areas were multiplied by two to account for both surfaces and quantified wax values are expressed as μg dm^{-2}.

2.4. Statistical analyses

The Camelina leaf wax constituents were analyzed using general linear models procedure (PROC GLM) of SAS software as randomized complete block experimental designs with replicates and accessions considered as random effects (Statistical Analysis System, SAS institute, 2001). The Correlation Coefficients (r) were used to assess the relationship between wax constituents, and was estimated using the PROC CORR of SAS. Broad-sense heritability on an entry-mean basis was calculated as $h^2 = (\sigma^2_{line}/(\sigma^2_{line} + \sigma^2_e/r)$ (Holland et al., 2003; Nyquist and Baker, 1991), where σ^2_{line} equaled the genetic variance among the Camelina accessions, σ^2_e is the variance of experimental error, and r is the number of replications.

3. Results and discussion

To date, there are few reports describing the variation in wax accumulation on Camelina organs (Razeq et al., 2014) and/or Camelina species (Tomasi et al., 2017). Accumulating cuticular wax on plant surfaces proves to be a strategy to reduce nonstomatal water loss under abiotic stresses (Fang and Xiong, 2015; Riederer and Schreiber, 2001). To extend the understanding of leaf wax-accumulating mechanisms and candidate genes controlling wax biosynthetic pathways in Camelina, large populations or diversity panels are required. The analysis of a spring diversity panel containing 163 accessions from different geographical areas revealed wide variations in wax constituents (Table 1; Fig. 1). The Camelina diversity panel showed a wide range of total wax contents, with the highest in an accession from Poland, which accumulated 288.1 μg dm^{-2} of total wax, and the lowest was an accession from Spain, with 148.8 μg dm^{-2} (Table 1). In general, wax compositions were similar to that previously identified in Camelina species (Tomasi et al., 2017), where the accessions exhibited a wide range of primary fatty alcohols, alkanes, wax esters, aldehydes, free fatty acids, alkylguaiacols, methylalkylresorcinols and β-sitostrol (Table 1). Together, primary alcohols, alkanes and wax esters accounted for 86% of the total wax content in the Camelina diversity panel. Primary alcohols contributed 35% of total waxes (Table 1), where Camelina accessions showed wide distribution of primary alcohols, ranging from 31.39 μg dm^{-2} to 119.1 μg dm^{-2}. Primary alcohols previously showed an increase in stressed soybean (Kim et al., 2007b) and maritime pine (Le Provost et al., 2013) leaves. Among primary alcohols in Camelina, C_{24}, C_{26} and C_{28} were the predominant molecular species that together counted for 84% of total primary alcohols (Table 1). In contrast, primary alcohols were not as prominent in *Arabidopsis* leaf wax (Bernard and Joubès, 2013), suggesting that the *Arabidopsis* wax biosynthetic pathway is differentially regulated in comparison to the Camelina wax biosynthetic pathway. Alkanes are abundant wax constituents that