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Chemical variation for leaf cuticular waxes and their levels revealed in a diverse panel of *Brassica napus* L.

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ABSTRACT

Brassica napus L. is one of the most important oilseed crops in the world, providing oil and protein used for food, fuel, and industrial purposes. Despite high oil yields and desirable agronomic traits, its geographical range is mainly limited to temperate climates, and oil yields and quality are negatively impacted by drought and heat stress. Leaf cuticular waxes are known to protect plants from many forms of environmental stress, including those caused by drought and heat. To shed light on the wax phenotypic diversity in *B. napus*, we quantified the levels of 24 leaf cuticular wax chemical constituents, and seven of their sums, in a diverse panel of 517 accessions representing *B. napus* seed stock center collections worldwide. Most of the 31 traits had moderately high heritability (H^2 = 0.19–0.81), suggesting that the observed phenotypic variation was influenced primarily by genetic effects. Further, we obtained a strong positive correlation between the two major branches of the metabolic pathway responsible for cuticular waxes. Although this metabolic linkage has been suggested by previous studies, it has not yet been statistically supported. We observed high correlations among individual alkane, secondary alcohol, and ketone constituents, and low correlations among individual primary alcohol and ester constituents. This study is the most extensive analysis of wax chemical diversity within any plant taxon to date, and lays a foundation for future studies of wax metabolism and function, and the application of new breeding strategies to modify leaf waxes and improve stress tolerance in *B. napus*.

1. Introduction

Brassica napus L. is a globally important crop whose seed oils are used for food, biofuels, lubricants, surfactants, and many other specialty products (Singh et al., 2008; Rahman 2013). Spring- and winter-growth habit types of *B. napus* are cultivated across the globe, wherein they encounter a multitude of diverse environments (Gunasekera et al., 2006; Rahman 2013). Over 36 million hectares of *B. napus* were planted in the 2012–2013 season worldwide (USDA FAS). In the United States, production of *B. napus* is increasing due to its importance as both an oilseed and bio-diesel crop (Singh et al., 2008), with 688 ha planted and harvested in 2013, and 1052 ha planted in 2014. The production of canola in the United States, which includes cultivars of *B. napus* developed for food and feed purposes (Savero, 1993), has increased from 545,516 ha planted in 2013 to 692,616 ha planted in 2014 (USDA NASS). *B. napus* is adapted to temperate environments and growing the crop under high temperature and water deficit conditions is associated with a significant reduction in seed oil content and increase in the production of glucosinolates (Aksouh et al., 2001). Depending on its intended use, the presence of glucosinolates in *B. napus* can be either beneficial (e.g., when conferring plant defense to phytophagous insects or serving as anticancer agents in the human diet) or detrimental (e.g., high levels have toxic or anti-nutritional impacts in the human diet, and are a contaminant in biofuel production). Improving the stress tolerance of *B. napus* in

Abbreviations: ASSYST, associative expression and systems analysis of complex traits in oilseed rape/canola; BLUP, best linear unbiased predictor; Co-A, coenzyme-A; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; GWAS, genome-wide association study; ITSD, internal standard; QTL, quantitative trait loci; SD, standard deviation of the BLUPs; SE, standard error of the heritabilities.

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temperate environments and widening its adaptive range would allow for increased cultivation and stabilization of oilseed supply, and improved crop performance in the face of climate change.

Genetic analysis has shown that heat and drought tolerance are heritable traits in *B. napus* (Hossein et al., 2012; Singh et al., 2008; Zakirullah et al., 2000). Although leaf cuticular waxes have been shown to confer these forms of stress tolerance in various plant species (Jenks et al., 1995, 2007; Kosma et al., 2009), the extent to which intra-specific variation in plant waxes occurs, its actual contribution to stress tolerance, and its heritability, has yet to be fully elucidated. Plant cuticular waxes are typically comprised of a heterogeneous mixture of long chain hydrocarbons, including alkanes, primary and secondary alcohols, aldehydes, ketones, and esters (Jenks et al., 1995; Jetter et al., 2007). In *B. napus*, the 29-carbon length alkane is the major wax constituent (Holloway et al., 1977). Based on inferences from studies in model systems, the preponderance of alkanes on *B. napus* leaves indicates that water conservation (alkanes create effective hydrophobic barriers) may be of adaptive significance for this species, although other explanations for the abundance of *B. napus*' C_{29} alkanes cannot be ruled out (Jenks et al., 2007; Kosma et al., 2009; Xu et al., 2014).

Previous studies have used genetic approaches to modify cuticular waxes to improve drought tolerance traits in plants (Lu et al., 2012; Seo and Park 2011; Zhou et al., 2014). For example, ectopic expression of wax-associated transcription factors in transgenic plants was shown to increase wax amounts and confer improved tolerance to water deficiency stress in some species (Seo and Park 2011; Zhou et al., 2014). Similar forms of stress tolerance could likely be achieved using traditional breeding approaches, wherein cultivars selected for cuticular wax properties that confer tolerance are used to introgress such desirable wax traits into elite lines. Quantitative trait loci (QTL) associated with waxes on the leaves of wheat, scored by visual leaf surface wax deposition (glaucousness), were found to explain 50% of the genetic variance in this trait, and the potential for selective breeding to optimize glaucousness was discussed (Bennett et al., 2012; Shepherd and Griffiths, 2006). Despite this, breeding for leaf cuticular wax chemical composition has not been reported, potentially due to limited knowledge about the compositional diversity of leaf waxes within potential breeding populations, and also perhaps due to the analytical challenges inherent in such extensive wax compositional studies.

The largest intra-generic plant population previously screened for variation in cuticular wax chemical composition occurred in Capsicum (fruiting pepper; Parsons et al., 2013), where it was found that total fruit wax amounts varied more than 16-fold across a group of 50 diverse accessions (Parsons et al., 2013). A comparable study of an intra-specific population of *Arabidopsis thaliana* [Brassicaceae] revealed an approximate 2-fold variation in leaf wax amount among 40 ecotypes, as well as significant variation for specific chemical constituents (Rashotte et al., 1997). In the study presented here, we assessed intra-specific variation for leaf wax composition in another member of the Brassicaceae, this time for 517 *B. napus* accessions from a worldwide collection (a diversity panel). We examined the amounts of 24 individual leaf wax constituents, and 7 derivative traits, to assess overall wax phenotypic variation within the *B. napus* species, and as a basis to quantify the influence of genetic effects on this variation. We reveal moderately high broad-sense heritability on an entry-mean basis for wax traits evaluated within the sampled *B. napus* diversity panel, suggesting that much of the variation is genetically determined. Challenges faced in such high throughput analysis of leaf surface wax content, which must underpin future studies of wax variation in diverse germplasm, are discussed. This study lays the foundation for future efforts to better understand genetic regulation of *B. napus* cuticular wax metabolism and ecological function, and for the application of new breeding strategies to modify leaf waxes to increase the environmental stress tolerance of *B. napus*, decrease its irrigation requirements (and achieve more crop per drop), and expand the geographical range where the *B. napus* oilseed crop can be profitably grown.

2. Materials and methods

2.1. Plant material and experimental design

The field trial was conducted at the Maricopa Agricultural Center of the University of Arizona in Maricopa, AZ. Replicates one and two of the *B. napus* panel were planted on February 7, 2013 and replicate three was planted on February 12, 2013. An Arizona Meteorological Network weather station located at 33°04′07″N, 111°58′18″W was approximately 2 km from the field site. Standard agronomic and pest control practices for flood irrigated crop production in the southwestern United States were implemented. The soil is a Casa Grande sandy loam (fine-loamy, mixed, superactive, hyperthermic Typic Natrargids).

The panel of 520 *B. napus* accessions (Supplementary Table 1) consists of 351 spring, 75 semi-winter, 39 winter types, and 55 with unknown growth types, where growth habit information was provided by each of the donating institutions. This *B. napus* collection was constructed to significantly cover the available genetic diversity of spring-type *B. napus* in public breeding programs from around the world. Semi-winter accessions that have a record of flowering without vernalization were also included (*personal comm.* John McKay). Accessions were obtained from the USDA-ARS North Central Regional Plant Introduction Station (NCRPIS) in Ames, Iowa, a component of the National Plant Germplasm System, the ASSYST panel (Körber et al., 2012), and the Australia Temperate Field Crops Collection. The semi-winter lines were originally from Wei Qian at Southwest University, China, and were provided by John McKay from Colorado State University. The panel was arranged as a 13×40 incomplete block α-lattice design (Patterson and Williams, 1976) with three randomized replications for a total of 1560 plots. Experimental units were one-row plots. Plots were 3.05 m in length with a 1.5 m alley at the end of each plot. Inter-row spacing was 1.02 m. In addition, one-row plots of *B. napus* accession UISC00.3.8.DE (spring) were planted on all sides of each replicate to reduce border effects.

2.2. Extraction and analysis of cuticular waxes

Of the 520 lines planted, seeds of three lines failed to germinate. The cuticular wax composition of leaves from all replicates of the remaining 517 lines was determined as described by Chen et al. (2003) with slight modifications. Briefly, uniform, fresh, fully expanded leaf samples, from south facing, fully sun exposed leaves, from all plots were collected nine weeks after planting and pre-flowering for all accessions. Sampling was done over the course of three days, with one biological replicate sampled each day. Sampling was performed on March 15 (replicate 1), 16 (replicate 2), and 17 (replicate 3) of 2013. Monthly temperatures averaged at 29 °C at time of sampling (http://ag.arizona.edu/azmet/data/0613em.txt, accessed 21.07.15.). A single leaf was collected from the center plant from each of the 1560 plots. Four leaf disc samples were taken from each single leaf collected using a 13 mm circular leaf punch (Humboldt #7 cork borer). Collected leaves were put on ice and taken to the lab for immediate wax extraction. The four leaf discs from each biological replicate were submerged in 3 ml of gas chromatography (GC)-grade hexane (Fluka) and agitated for 45 s in a 20 ml standard glass scintillation vial, and then the hexane was transferred to a new vial. The tissues were rinsed with additional hexane for approximately 1 s, then the hexane fractions were com-

Table 1
Means and ranges (in $\mu g\,dm^{-2}$) for untransformed BLUPs of 31 cuticular wax traits evaluated in a *B. napus* diversity panel and estimated broad-sense heritability on an entry-mean basis in a single environment in Maricopa, Arizona. The table is organized by wax classes. Within each wax class, values are sorted by heritability. BLUPs: best linear unbiased predictors; SD: standard deviation of the BLUPs; SE: standard error of the heritabilities; Total Wax Content, summed value of all 24 wax traits; primary alcohols, total primary alcohols; acids, total acids; alkanes, total alkanes; esters, total esters; alkane branch of the pathway, C_{29} alkane, C_{29} secondary alcohol, C_{29} ketone; primary alcohol branch of the pathway, C_{26}, C_{28}, C_{30} alcohols and 43, 44, 45, and 46 esters.

		BLUPs			Heritabilities	
Trait	No. lines	Mean	SD	Range	Estimate	SE
C_{24} alcohol	415	1.21	0.54	0.66–5.90	0.63	0.03
C_{29} alcohol	504	4.89	0.78	2.18–6.64	0.56	0.04
C_{30} alcohol	504	4.69	0.73	3.14–6.59	0.54	0.04
C_{28} alcohol	504	13.35	2.38	9.18–21.93	0.53	0.04
C_{26} alcohol	504	17.15	4.07	11.63–31.66	0.45	0.04
C_{29} alcohol-2	504	81.82	12.06	70.27–96.39	0.19	0.07
C_{28} acid	504	11.38	2.89	4.50–33.94	0.75	0.02
C_{26} acid	514	2.41	0.78	0.79–7.01	0.72	0.02
C_{30} acid	503	44.88	10.02	19.19–95.77	0.62	0.03
C_{29} alkane	504	677.30	71.00	285.94–892.38	0.68	0.03
C_{30} alkane	503	8.58	1.33	4.26–13.20	0.65	0.03
C_{27} alkane	516	3.18	0.72	1.78–6.36	0.62	0.03
C_{28} alkane	503	3.25	0.55	2.11–5.07	0.56	0.04
C_{31} alkane	517	40.51	10.46	26.62–72.58	0.47	0.04
43 ester	502	11.71	2.37	5.33–23.20	0.66	0.03
45 ester	502	14.75	2.94	5.58–26.68	0.65	0.03
46 ester	502	23.21	5.17	9.39–37.85	0.58	0.03
44 ester	502	26.06	6.17	11.02–43.59	0.58	0.03
C_{29} ketone	504	421.11	51.89	200.35–574.4	0.61	0.03
29-diketone	504	102.72	16.18	55.26–145.58	0.53	0.04
C_{28} aldehyde	515	8.51	2.08	3.59–15.36	0.52	0.04
26-methyl-octacosanol	503	100.94	14.52	45.89–147.45	0.72	0.02
24- methyl-hexacosanol	504	43.67	8.18	17.08–85.43	0.72	0.02
24-methyl-pentacosanol	503	4.12	1.03	2.57–7.19	0.45	0.04
Total wax content	517	161.94	98.20	686.82–2255.30	0.70	0.03
Primary alchohols	502	75.52	5.08	31.10–125.47	0.61	0.03
Acids	504	41.49	0.10	30.23–66.49	0.46	0.04
Alkanes	514	59.71	2.51	30.69–138.08	0.70	0.03
Esters	517	718.80	88.05	302.80–970.00	0.61	0.03
Alkane pathway	517	1211.05	49.30	519.10–1622.90	0.81	0.02
1-Alcohol pathway	505	111.19	6.32	61.67–167.79	0.75	0.03

bined. The volume of hexane-soluble cuticular wax extracts was reduced under nitrogen until the samples could be transferred into 500 μl GC glass insert vials. The scintillation vials were given an additional rinse of hexane, which was transferred to the appropriate GC insert vial, and then samples were evaporated to dryness under nitrogen.

The gas chromatography–mass spectrometry (GC–MS) analysis of cuticular wax content and composition was performed using an Agilent 7890A GC and 5975C Triple-Axis detector Mass Spec with 12 m, 0.2 mm HP-Ultra 1 capillary column with helium as the carrier gas. The internal standard hexadecane was added to the evaporated samples in the sample vials and waxes were derivatized by heating in N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) at 80 °C for 30 min prior to injection. The column temperature was programmed with an initial temperature of 80 °C and increased 18 °C/min to 245 °C and held for 4.8 min, then increased 19 °C/min to 325 °C and held for a final 11.8 min. The molecular identities of individual wax compounds were determined by quadrupole electron impact GC–MS, using relative retention time and mass fragment spectra of each molecular species and comparison to the NIST MS Search 2.0 database, as well as comparison of the spectra to *bona fide* standards run on the same instrument. The calculated wax profiles were generally similar to that reported by Holloway et al. (1977). The quantification of each compound was based on peak areas relative to the internal standard (ITSD) hexadecane. Correction factors were determined using external calibration curves generated using standards for a subset of the major alcohols, alkanes, and acids. The amount of cuticular wax includes both the adaxial and abaxial leaf surfaces, and values are expressed per unit of leaf surface area ($\mu g\,dm^{-2}$).

A large subset of leaf samples was extracted and then also processed using the Agilent 7696A Sample Prep WorkBench. Although the instrument allowed for sample prep automation, mechanical errors and failures of the injection process decreased accuracy and required regular monitoring. While a promising method for future studies, the ability of the instrument to aid in such high-throughput phenotyping of waxes as performed in this study was, and is yet, limited in our hands.

2.3. Statistical analysis

For the collected wax profiles, each individually measured compound was assessed as a single trait. Seven derivative traits representing compound classes or pathways, each calculated as the sum of several individual compounds, were also considered. A complete list of the 31 traits analyzed is provided in Table 1. Outliers were removed using Studentized deleted residuals (Kutner et al., 2004) in SAS version 9.3 (SAS Institute 2012; Littell et al. (1996)) using mixed linear models that included random effects accounting for variation in the field, laboratory, and among the lines. For each trait, a best linear unbiased predictor (BLUP) of each line was obtained from a mixed linear model fitted across all replicates in ASReml version 3.0 (Gilmour et al., 2009):

$$Y_{ijklmn} = \mu + \text{line}_i + \text{replicate}_j + \text{block(replicate)}_{jk} + \text{column(replicate)}_{jl} + \text{GC–MS\_run\_method}_m + \text{GC–MS\_run\_date}_n + \varepsilon_{ijklmn}, \quad (1)$$

in which Y_{ijklmn} is an individual phenotypic observation, μ is the grand mean, line_i is the effect of the ith line, replicate_j is the effect

of the jth replicate, block(replicate)$_{jk}$ is the effect of the kth block within the jth replicate, column(replicate)$_{jl}$ is the effect of the lth column within the jth replicate, GC–MS_run_method$_m$ is the effect of the mth GC–MS run method, GC–MS_run_date$_n$ is the effect of the nth GC–MS run date, and ε_{ijklmn} is the random error term. The optimal model for each trait was determined using a backwards model fitting procedure that has been previously described (Chandler et al., 2013). The final model for each trait was used to estimate the BLUPs for each line and variance components. These variance components were used to estimate broad-sense heritabilities on an entry mean basis (H^2) or repeatability (Piepho and Möhring, 2007) per the method of Holland et al. (2010). The value of H^2 ranges from 0 to 1, with higher values indicating that a larger proportion of phenotypic variation is attributable to genetic factors. The delta method was used to approximate the standard error of the heritability estimates (Holland et al., 2010). Finally, the line BLUPs from each trait were screened for outliers using Studentized deleted residuals and summary statistics are included in Table 1. Pearson's correlation coefficients (r) were used to assess the relationship between untransformed cuticular wax trait BLUPs. We also conducted a principal component analysis (PCA) of the trait BLUPs with the *prcomp* function in R version 3.2.1 (R Core Team, 2015).

3. Results

3.1. Chemical composition of cuticular wax

A total of 517 lines were analyzed to characterize the leaf wax content and composition within a diverse collection of *B. napus* germplasm. Waxes were identified and quantified using GC–MS (Fig. 1), and all accessions contained cuticular waxes consisting of aldehydes, primary alcohols, secondary alcohols, unbranched straight chain alkanes, branched methyl alcohols, ketones, diketones, and esters. The general profile of the 24 wax compounds in the diversity panel is similar to that reported by Holloway et al. (1977), who previously characterized wax content in a small set of three *B. napus* lines. The overall composition of the cuticular wax profile is also similar to other *Brassicaceae*, such as *A. thaliana* or *Brassica oleracea*, which also typically have the dominant constituents of C_{29} alkane, C_{29} ketone, and C_{29} secondary alcohols (Baker, 1974). One difference in our data set from the Holloway et al. (1977), study, however, was our identification of a significant molecular species (peak #13) that exhibited a mass spectrum consistent with a diketone, with diagnostic ions at m/z 241, 227, 213, 125, 111, 97, 83, 69, 57 and 43 (Supplementary Fig. 1). To test whether this compound was consistent with this molecular structure, two test samples were run, one derivatized with BSTFA, which modifies free hydroxyl groups, and an underivatized sample. The resulting chromatograms showed no change in relative retention time of the unknown compound (data not shown), suggesting the compound was not an alcohol, fatty acid or ketol. While these results are consistent with a diketone structure, further experiments are needed for more definitive structural identification.

The two most abundant compounds that we detected in these 517 *B. napus* lines (Supplementary Table 1) were the C_{29} alkane and the C_{29} ketone, whereas the C_{24} alcohol was the least abundant of those quantified (Table 1). Together, the two major constituents of *B. napus* wax account for up to 65% of the total wax content, which ranged from 687 to 2255 $\mu g\,dm^{-2}$. For wax classes, n-alkanes ranged from 31 to 138 $\mu g\,dm^{-2}$, acids from 30 to 66 $\mu g\,dm^{-2}$, and esters ranged from 302 to 970 $\mu g\,dm^{-2}$. There was also a large range for the amounts of the major chain lengths within the predominant wax classes (Table 1). Notably, the highest C_{29} alkane amount was 892 $\mu g\,dm^{-2}$ and the lowest C_{29} alkane amount was 286 $\mu g\,dm^{-2}$. Additionally, the highest C_{29} ketone amount was 574 $\mu g\,dm^{-2}$, and the lowest amount was 200 $\mu g\,dm^{-2}$.

A strong positive correlation was found between the C_{29} alkane and C_{29} ketone wax components as measured by Pearson's correlation coefficient ($r = 0.73$). Over all 24 measured components, large correlation values were observed for a number of cuticular wax traits (Supplementary Table 2), a possible indication that not all of these wax compounds are independently regulated (Fig. 2). In the metabolic pathway, the correlations between products in the branch leading to alkanes and their derivatives (C_{29} alkane, C_{29} secondary alcohol, and the C_{29} ketone; Fig. 2) were all high, averaging around $r = 0.60$. These correlations suggest that the compounds present within this branch of the pathway are highly coupled with one another. In contrast, large correlations were not observed in the pathway represented by the conversion of primary alcohols to esters (primary alcohol ester branch; C_{26}, C_{28}, C_{30} alcohols and 43, 44, 45, and 46 esters). The correlation between the pathway branch containing the C_{29} alkane and its derivatives and the branch containing primary alcohols and esters was high ($r = 0.75$), indicating these two pathway branches are also highly coupled, despite the low correlations found among individual components in the primary alcohol and ester pool. Interestingly, no distinct major patterns were revealed by the first two PCs that explained ~94% of the variance obtained from a PCA conducted on all 31 leaf cuticular wax traits (results not shown).

3.2. Heritability measurements of the wax traits

To evaluate the potential response to selection of the measured wax traits through breeding programs, the broad-sense heritability on an entry-mean basis was calculated for all 31 traits. Heritability values ranged from 0.19 (C_{29} secondary alcohol) to 0.75 (C_{28} acid) for each independent wax trait (Table 1). The heritabilities calculated for the components in the alkane and its derivatives pathway branch, and for the primary alcohol ester branch were also moderately high ($H^2 = 0.81$ and $H^2 = 0.75$, respectively). Heritability estimates obtained for most of these compounds suggest that the variation for cuticular wax traits is influenced mainly by genetic rather than environmental factors and that these traits are likely suitable for a genome-wide association study (GWAS) to identify the major genetic factors controlling quantitative variation in leaf wax traits.

4. Discussion

These data reflect the largest analysis of cuticular wax chemical composition in a single plant taxon to date, and provide extensive leaf cuticular wax phenotypic data representing a large diversity panel of *B. napus*. The primary components of *B. napus* cuticular wax include alkanes, aldehydes, primary and secondary alcohols, ketones, and esters, and these are quite consistent with those of previous studies by Holloway et al. (1977). The diversity panel of 517 accessions represents a broad range of geographical, and to some degree, ecological regions for *B. napus*, for spring-type *B. napus* in particular. Our results showed that the 517 *B. napus* accessions possessed a similar range of variation for wax amount as was reported for the 40 *A. thaliana* ecotypes (Rashotte et al., 1997), about 2-fold, but much less variation than reported among 50 *Capsicum* accessions (Parsons et al., 2013). In no previous studies however has the heritability of wax composition been determined. By estimating heritabilities, we demonstrate how sources of genetic variation underlying leaf wax amounts in *B. napus* could be elucidated though GWAS, and likely harnessed in breeding programs with a goal to improve *B. napus* stress tolerances.

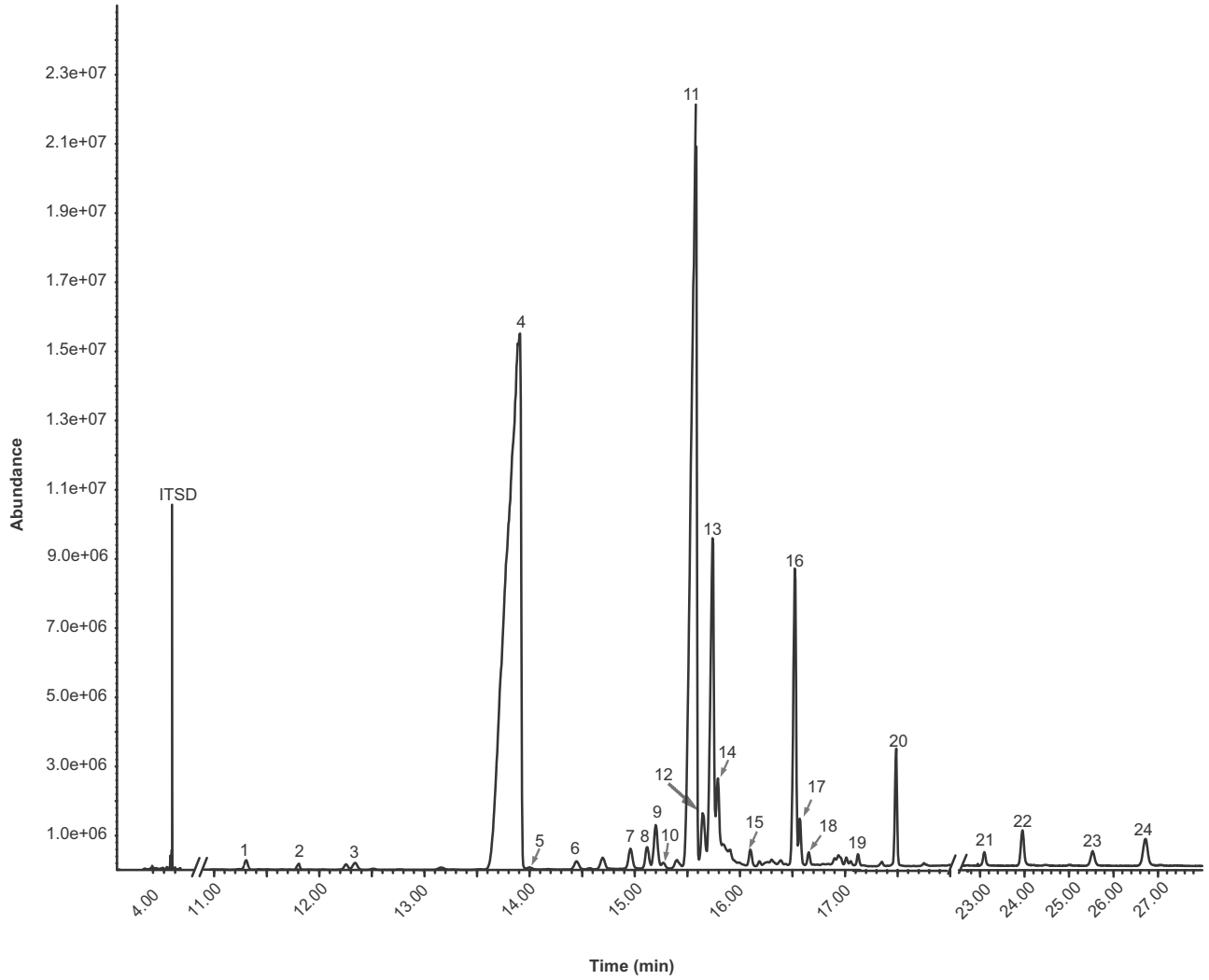

Fig. 1. GC–MS chromatogram of hexane soluble cuticular wax components obtained after *N*,*O*-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) derivitization from a representative individual of the *B. napus* diversity panel. ITSD: internal standard hexadecane (5 μg). (1) C_{27} alkane, (2) C_{24} alcohol, (3) C_{28} alkane, (4) C_{29} alkane, (5) 24-methyl pentacosanol, (6) C_{26} alcohol, (7) C_{30} alkane, (8) C_{28} aldehyde, (9) 24-methyl hexacosanol, (10) C_{26} acid, (11) C_{29} ketone, (12) C_{29} alcohol-2, (13) C_{29} diketone, (14) C_{31} alkane, (15) C_{28} alcohol, (16) 26-methyl octacosanol, (17) C_{28} acid, (18) C_{29} alcohol, (19) C_{30} alcohol, (20) C_{30} acid, (21) 43 ester, (22) 44 ester, (23) 45 ester, (24) 46 ester.

Relatively high amounts of *n*-alkanes, specifically the C_{29} alkane, were uniformly observed throughout the population of *B. napus*. This suggests that the inhibition of leaf water loss via reduced cuticle permeability may be an important trait in *B. napus*, as alkanes have been specifically linked to this function in previous studies (Leide et al., 2007; Parsons et al., 2013; Vogg et al., 2004). The heritability of the C_{29} alkane is relatively high ($H^2 = 0.68$), as compared to the other identified compounds, signifying a large genetic component to this trait. Whether these alkanes function in drought tolerance, or another ecological function, the high heritability of the C_{29} alkane suggests it as a potential target for plant breeders. The C_{29} alkane is a precursor for the generation of the C_{29} ketone, with secondary C_{29} alcohol as the intermediate (Fig. 2; Greer et al., 2007). A high correlation is observed between the C_{29} alkane and secondary C_{29} alcohol ($r = 0.55$), the secondary C_{29} alcohol and the C_{29} ketone ($r = 0.50$), and also the C_{29} alkane and C_{29} ketone ($r = 0.73$), affirming using statistical analysis that the components produced by the wax pathway are metabolically linked (Table 1). The heritability values of the C_{29} alkane and C_{29} ketone are also high, $H^2 = 0.68$ and $H^2 = 0.61$, respectively, however, the heritability of the secondary C_{29} alcohol is the lowest of all the observed traits at $H^2 = 0.19$. The observed low heritability for the C_{29} alcohol abundance is difficult to explain at this time. Perhaps the actual size of the C_{29} secondary alcohol pool on the leaf surface is less critical ecologically, with the observed high fluctuation in its pool size more a result of required flux in the pathway to maintain optimum C_{29} alkane and ketone levels. Further study is needed, both on the ecological role of these compounds, and the cellular and environmental factors that influence flux in these wax metabolic pathways.

We observed a strong positive correlation ($r = 0.75$) between the total abundance of the alkane pool and primary alcohol pool. As such, the alkanes and primary alcohols, representing the two main branches in the wax pathway (Fig. 2), are not only coupled with one another, but also highly heritable. This association suggests that the shunting of early precursors (probably acyl-CoAs) toward either the pathway branch containing alkanes or else the branch containing primary alcohols is likely an important point of regulatory control in wax metabolism. Interestingly, the main compounds that make up the primary alcohol to ester branch of the pathway are not highly correlated with one another, whereas strong correlations were observed among compounds within the branch of the pathway containing alkanes (*Supplementary* Table 2). A metabolic or ecological explanation for the differences observed in these pathway branches is not yet evident. Notwithstanding,

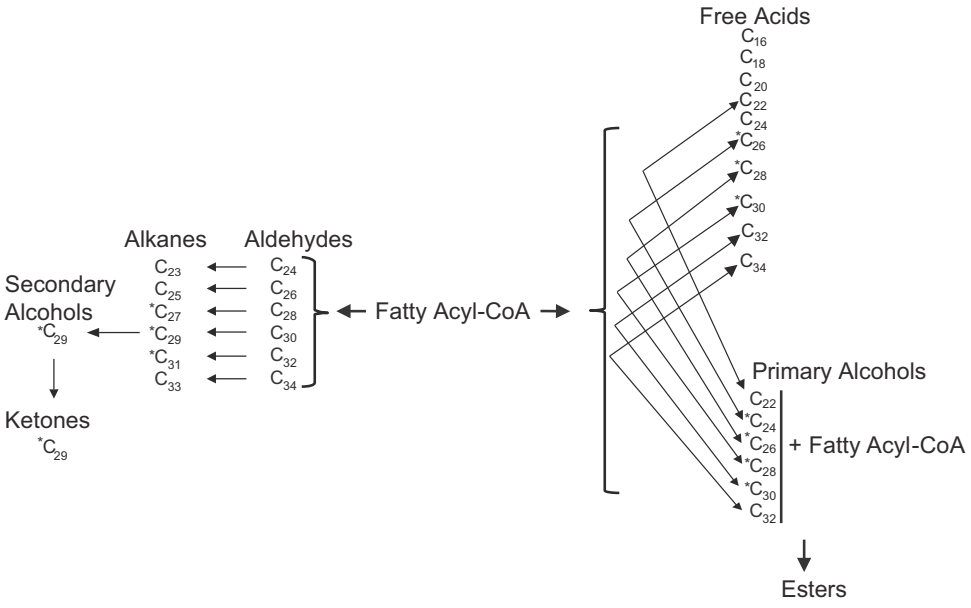

Fig. 2. Pathway of leaf cuticular wax biosynthesis in *B. napus*. Compounds identified in our analyses and found in the wax biosynthesis pathway are demarcated with an asterisk (*). The diketones and methyl branched constituents assessed in this report are not included in the figure. CoA: Coenzyme A.

our results indicate that major wax components within each of the main pathway branches could be ideal targets for breeding programs, and that approaches like GWAS will likely provide additional insight into important genes and regulatory determinants associated with these pathway branches.

There are well-known relationships between plant waxes and plant biotic and abiotic stress tolerances (Dodd and Afzal-Rafii 2000; Jenks et al., 2000, 2007), and a rigorous understanding of the extent to which both genetics and environment play a role in determining wax amount and composition is needed to inform a genomics-assisted breeding strategy whose goal it is to modify waxes. Similar to our findings, Dodd and Afzal-Rafii (2000) identified a strong genetic component to the variation observed in cuticular wax composition in three species from the Cupressaceae family (*Austrocedrus chilensis*, *Fitzroya cupressoides*, *Pilgerodendron uviferm*) by calculating broad-sense heritability. Even though the variation in our traits appears to be largely attributable to genetic factors, both the environment and interaction between genotype and environment will surely contribute to phenotypic variation (Kosma et al., 2009, 2010; Seo and Park 2011; Zhou et al., 2014). Further, observing the rate of change in the amount and composition of wax in response to environmental stress might allow wax metabolic responsiveness itself to be dissected in GWAS. Without a doubt, the identification of genetic determinants of stress tolerance, especially heat and drought tolerance, is important for improving crop resilience to changing and stressful environments.

5. Conclusions

This study reveals the presence of significant heritable variation in cuticular wax amount and composition within a large and diverse *B. napus* panel of 517 accessions. Lines from this panel have potential for use as parental lines in breeding efforts focused on modifying leaf waxes, and through genomics analysis the identification of genes associated with valuable wax traits. The moderate to high heritability of various *B. napus* wax traits indicates they are excellent candidates for a GWAS and may respond favorably to genomic selection in plant breeding programs. High heritability of the predominant C_{29} alkane constituents suggests that selection for alkane amount may be effective in breeding programs to improve *B. napus* response to abiotic stress, but especially drought stress.

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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.2015.10.047.

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