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ORIGINAL ARTICLE

Characterization and genetic mapping of the β-diketone deficient *eceriferum-b barley* mutant

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Abstract
Key message **The barley *eceriferum-b.2* (*cer-b.2*) mutant produces glossy leaf sheaths and is deficient in the cuticular wax component 14,16-hentriacontanedione. The mutated gene maps to a 1.3-cM interval on chromosome 3HL flanked by the genes MLOC_10972 and MLOC_69561.**

Abstract The cuticular wax coating of leaves and stems in many grass species is responsible for the plants' glaucous appearance. A major component of the wax is a group of β-diketone compounds. The barley *eceriferum-b.2* (*cer-b.2*) mutant produces glossy leaf sheaths and is deficient for the compound 14,16-hentriacontanedione. A linkage analysis based on 708 gametes allowed the gene responsible for the mutant phenotype to be mapped to a 1.3-cM interval on chromosome 3HL flanked by the two genes MLOC_10972 and _69561. The product of the wild type allele may represent a step in the β-diketone synthesis pathway.

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Introduction

All aerial organs of plants are covered by the cuticle: leaf and stem of plants are covered by the waxy cuticle, which restricts water loss and forms a physical barrier against pathogens (Bernard and Joubès 2013; Kunst and Samuels 2009; Riederer and Schreiber 2001; Xu et al. 1997). Some of the wax is deposited outside the cuticle (epicuticular wax) and some within it (intracuticular wax) (Bernard and Joubès 2013; Broun et al. 2004; Jenks et al. 2002; Jetter and Schäffer 2001). The epi- and intracuticular waxes are responsible for the glaucous appearance of the surface by refracting the incident light (Bianchi and Figini 1986; Jenks et al. 2002; Jetter and Schäffer 2001). The chemical constitution of cuticular wax comprises very long chain (C_{20}–C_{34}) fatty acids, primary and secondary alcohols, aldehydes, alkanes, ketones and alkyl esters (Kunst and Samuels 2003; Li-Beisson et al. 2010; Yeats and Rose 2013). The precursor fatty acids of chain length C_{16}–C_{18} are synthesized by fatty acid synthase in the plastids, from where they are exported to the cytoplasm and elongated in the endoplasmic reticulum (Bernard and Joubès 2013; Kunst and Samuels 2003, 2009). The barley C_{29}–C_{33} β-diketones are synthesized by an enzyme complex which features of both fatty acid elongase and polyketide synthase (PKS) (Mikkelsen 1979; Schneider et al. 2016; von

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Wettstein-Knowles 1972, 1980, 1986, 1992, 2012). The predominant β-diketone is hentriacontan-14,16-dione (von Wettstein-Knowles 1980).

In barley, variation in spike and stem glaucousness is correlated with the amount of β-diketone present in the epicuticular wax (von Wettstein-Knowles 1972, 1986). Comparisons between glaucous and non-glaucous mutants (*cer-c*, *-q* and *-u*) have revealed that the wild type gene product is involved in the production of β-diketones, hydroxy-β-diketones, and alkan-2-ols (von Wettstein-Knowles 1986, 2012). In the *cer-c* mutant, the formation of β-diketones and hydroxy-β-diketones is blocked (von Wettstein-Knowles 1992); in *cer-q* the synthesis of β-diketones and their derivatives is compromised (Mikkelsen 1979); while in *cer-u* the accumulation of hydroxy-β-diketones is restricted, leading to an increase in the presence of β-diketones (von Wettstein-Knowles 1972). It has been shown that the *cer-c* mutation disrupts a gene encoding a chalcone synthase-like PKS, that the *cer-q* mutation represents a lesion in a gene encoding a lipase/carboxyl transferase and that the *cer-u* mutation has affected the function of a P450 enzyme (Hen-Avivi et al. 2016; Schneider et al. 2016).

The X-ray induced, recessive barley *eceriferum-b.2* (*cer-b.2*) mutant produces glossy spikes and leaf sheaths and glaucous leaf blades (Lundqvist and Wettstein 2009; Rasmusson and Lambert 1965). There are 1580 cuticular wax-deficient mutants, termed *eceriferum* (*cer*), in 79 complementation groups in barley (Lundqvist and Lundqvist 1988). In *cer-c, -q* and *-u* mutants the spikes and uppermost leaf sheaths are glossy, but the leaf blades are glaucous. The *Cer-b.2* involved in the mutation has been mapped to chromosome 3HL (Eslick and McProud 1974; Konishi 1970; Singh and Tsuchiya 1974). An introgression region of 17.2 cM carrying *cer-b.2* is identified in the near isogenic line BW107 (Druka et al. 2011). In this work, we characterized the effect of *cer-b.2* on the cuticular wax composition and located the mutated genomic region to a 1.3-cM interval on chromosome 3HL.

Materials and methods

Plant materials

The *cer-b.2* mutant, originally isolated in the cultivar Maja (Franckowiak and Lundqvist 2011), has been transferred by serial back-crossing into cv. Bowman to produce the near isogenic line BW107 (Druka et al. 2011), hereafter referred to as BW-NIL (*cer-b.2*). Grain of BW-NIL (*cer-b.2*) and of cv. Bowman (PI 483237) were obtained from the United States Department of Agriculture, Agricultural Research Service (USDA-ARS). A cross was made between the wild barley accession 23–19 (obtained from the Institute of Evolution, University of Haifa, Israel) and BW-NIL (*cer-b.2*). The resulting F_1 grains were germinated on moist filter paper and kept at 4 °C in the dark for 7 days, after which they were potted into soil in a greenhouse and allowed to self-fertilize.

Scanning electron (SEM) and transmission electron (TEM) microscopy

For SEM, leaf sheath segments of length ~1 cm were cut from the middle section of cv. Bowman and BW-NIL (*cer-b.2*) flag leaf sheaths, mounted on an aluminum stub using double-sided carbon tape, snap-frozen in liquid nitrogen and examined using a Hitachi S-3400N SEM device (http://www.hitachi-hightech.com) applying an accelerating voltage of 10 kV. For TEM, flag leaf sheaths of cv. Bowman and BW-NIL (*cer-b.2*) were cut prior to anthesis into 1 cm segments, rinsed twice for 10 min in 0.1 M phosphate buffer (pH 7.2), fixed overnight in 1.0% w/v OsO_4, re-rinsed in phosphate buffer, dehydrated by passing through an ethanol series (30–100%), followed by curing in acetone/epon812 (1:1) (12 h at 45 °C and 24h at 65 °C). The material was then sectioned (40–70 nm) using an LKB Ultramicrotome Model V microtome (LKB-Produkter, Bromma, Sweden), and the sections stained in 3% (w/v) uranyl acetate followed by 3% (w/v) lead citrate. The TEM scanning of the samples was achieved using a JEM-1230 device (Jeol Ltd. Tokyo, Japan). Cuticle thickness was estimated using Nano measurer 1.2 software (nano-measurer.software.informer.com).

Wax extraction

The flag leaf sheaths of three plants of each of cv. Bowman and BW-NIL (*cer-b.2*) were removed when the plants had reached anthesis. Their surface area was approximated by considering the sheath as a cone. Wax was extracted from the leaf sheath samples by immersion for 10 min (with occasional shaking) at room temperature in 15 mL chloroform containing 5 mg/mL *n*-tetracosane (Sigma–Aldrich, St. Louis, MO, USA; cat #87089) within a 20 mL glass tube sealed with a PTFE screw-cap lid. The chloroform was evaporated by passing nitrogen gas above the extract at room temperature. The extracted wax was then derivatized by the addition of 100 µL *N,O*-bis(trimethylsilyl) trifluoroacetamide (Supelco, Bellefonte, PA, USA; cat #3-3024) and 100 µL anhydrous pyridine (Sigma–Aldrich; cat #270970) at 80 °C for 1 h with vortexing every 15 min. The derivatized samples were finally dried under a stream of nitrogen gas and dissolved in 200 µL chloroform.

Gas chromatography-mass spectrometry (GCMS)

The constitution of the wax was derived by GCMS using a GC 6890N device (Agilent Technologies, Santa Clara, CA, USA) equipped with a 5973 inert mass selective detector. Each 2 µL injection was performed using a 10/1 split ratio. The inlet temperature was set to 250 °C, the carrier gas flow rate to 0.8 mL/min, the nominal inlet pressure to 11.35 psi. The oven temperature was set to start at 80 °C, then to increase by 4 °C/min until it reached 290 °C, where it was held for 25 min. The retention time locking feature was used, based on the retention time of the *n*-tetracosane internal standard (39.7 min). Total ion scans used 60–600 amu. The data were processed using Agilent GC Chemstation software (D.03.00) in conjunction with the National Institute of Standards and Technology mass spectral library (v8.0). Only peaks appearing after 38 min were considered, as there were no differences between cv. Bowman and BW-NIL (*cer-b.2*) before this time, and the earlier peaks were all relatively small. We changed the ordinate axis marked maximum abundance 2.8e+0.7 to 100, and divided the maximum value of 100 labeled on ordinate axis into five equal parts.

RNA extraction and transcriptome sequencing

Total RNA was isolated from the youngest leaf of three leaf stage seedlings of 23–19 and BW-NIL (*cer-b.2*) using an RNAprep pure Plant Kit (Tiangen, Beijing, China). The quality and quantity of the RNA were assessed using a Nanodrop 2100 instrument (Thermo Fisher Scientific, Wilmington, DE, USA). The poly-A mRNA was purified by magnetic Oligo (dT) beads and fragmented followed by cDNA synthesis. The cDNA fragments were cutting into blunt-ended and ligated to sequencing adaptors. The ligation products were then size selected for an insert size of 200 bp, and enriched by PCR with specific adaptor primers. Finally, the library was subjected to sequence by the Illumina HisSeq™ 2000 platform using 151 bp paired end reads. After removing low quality and adapter sequence, around 54 million filtered reads were obtained from 23–19 and around 17 million from BW-NIL (*cer-b.2*). Following a de novo assembly protocol, it was possible to recognize 87,655 unigenes in the 23–19 template and 38,276 in the BW-NIL (*cer-b.2*) template.

Linkage mapping

The F_2 population bred from the cross 23–19 × BW-NIL (*cer-b.2*) was used to generate a localized linkage map. The allelic status at the *cer-b.2* locus of each F_2 individual was deduced from the phenotype (glaucous vs glossy leaf sheath) of its F_3 progeny. As the *cer-b* locus is known to map to chromosome 3HL, this region was targeted with the barley genome zipper (Mayer et al. 2011), and its gene content (webblast.ipk-gatersleben.de/barley) used to search the set of BW-NIL (*cer-b.2*) and 23–19 unigenes for sequence polymorphisms between homologs. The single nucleotide polymorphism (SNP) assays used for genotyping the F_2 progeny are described below. We score segregation in the F_2 population by the SNP technology. The resulting linkage maps were constructed using JoinMap4.0 software (Kyazma 2006), applying the Kosambi mapping function (Kosambi 1944) to convert recombination frequencies into centiMorgans. MapChart software (Voorrips 2002) was used to draw the barley genetic maps. Use was made to the barley physical map (International Barley Genome Sequencing Consortium 2016) to identify additional candidates for the mutated gene.

SNP assay

Genomic DNA was extracted from fresh leaves of the F_2 progeny using an established method (Komatsuda et al. 1998), and used as the template for 10 µL PCRs. Each reaction contained 40 ng template, 1 µL 10× Ex Taq buffer, 25 mM $MgCl_2$, 2.5 mM dNTP, 10 µM of each primer (Table 1) and 0.375 U Taq polymerase (Takara Tokyo, Japan). The reaction was initially denatured by holding at 94 °C for 5 min, after which it was cycled 30 times through 94 °C/30 s, 60 °C/30 s, 72 °C/40 s, and then given a final extension step of 72 °C/5 min. The amplicons were digested for up to 3 h by the addition of 1 U of an appropriate restriction enzyme (Table 1), identified using the NEBcutter tool (nc2.neb.com/NEBcutter2). The restriction fragments were electrophoretically separated through a 1–3% (w/v) agarose gel, and visualized by EtBr staining.

Results

The *cer-b.2* mutation affects epicuticular wax, but not the cuticle membrane

The leaf sheaths of the upper leaves of cv. Bowman were glaucous while those of BW-NIL (*cer-b.2*) were glossy (Fig. 1). Inspection by SEM showed that the entire leaf sheath surface of cv. Bowman was covered by tubule-shaped structures, but these were absent from the BW-NIL (*cer-b.2*) leaf sheath surface, which instead harbored platelet-shaped structures (Fig. 2a, b). However, there was no evidence of any difference between the leaf sheath cuticle membrane in the two genotypes (Fig. 2c, d). The thickness of the cv. Bowman cuticle was 0.025 ± 0.005 µm, which was not significantly different from that of the BW-NIL (*cer-b.2*) cuticle (0.023 ± 0.005 µm).

Table 1 Primer sequences and restriction enzymes used for SNP genotyping

Marker	Primer F (5′–3′)	Primer R (5′–3′)	AT (℃)	Restriction enzyme
MLOC_5326	TGCAGCAGCTAGACCCTACC	ATTACTAACCATACTCTTCT	60	*Nco*I
MLOC_13723	CACGCCAGCATTCTCGTTA	TGGGATTGGAGGAGTTATGG	60	*Hin*fI
MLOC_12015	TTATCTTTGGCCCGAAACAC	CATCGATCCTGTCGTTTGC	60	*Bsa*HI
MLOC_12016	TTTGGCCCGAAACACTGAAG	TGGGACTACAAGCACTCTGG	60	*Bsa*HI
MLOC_36649	GCCACAAAGATTCATGGACA	GCGGCTGCTTGTTTACCTAT	60	*Msp*I
MLOC_10972	CAGCGGGAACAGTGGATAAT	TCTTGATGTTGGCTGTGGAG	60	*Hpy*CH4V
MLOC_69561	GGGCAGGTGCTACAGAAGAG	CAGAACTTCGGATCACAGCA	60	*Sma*I
MLOC_61206	CGAAGCTCGTGTCGTTGAT	TATCCGCACTCCCAAGCTAT	60	*Hpy*CH4V
MLOC_39313	AACGTGATGCCGATCTTGA	CTCCGGAACCGATTTCCTA	58	*Hpy*166II
MLOC_50	TGCCCAGTGAGATGTGTAGG	CGCTGTCGAGGATGTACGA	60	LP
MLOC_3646	GGCAAAACACCATCAAATCC	GGCAAGTACGTCATCCACAA	60	*Taq*I
MLOC_72373	CCGGTACTCATTTGGCAGTT	GGCTGGTTGGTTGAAGGATA	60	*Sca*I
AK364791	GTAGCTCAAGTGTGGCATCG	GCTGCACTTCCTCTGTCAAC	60	*Rsa*I
AK252399	TCAGTCTCGCAACCATCAAG	GTTCCGCCAGCTGCTCAT	60	*Sal*I
MLOC_16004	TGGACCTTCACACTTGTTCCT	CAATCCAATTCCACCAATGC	60	*Sna*BI

AT annealing temperature, *LP* length polymorphism, no enzyme

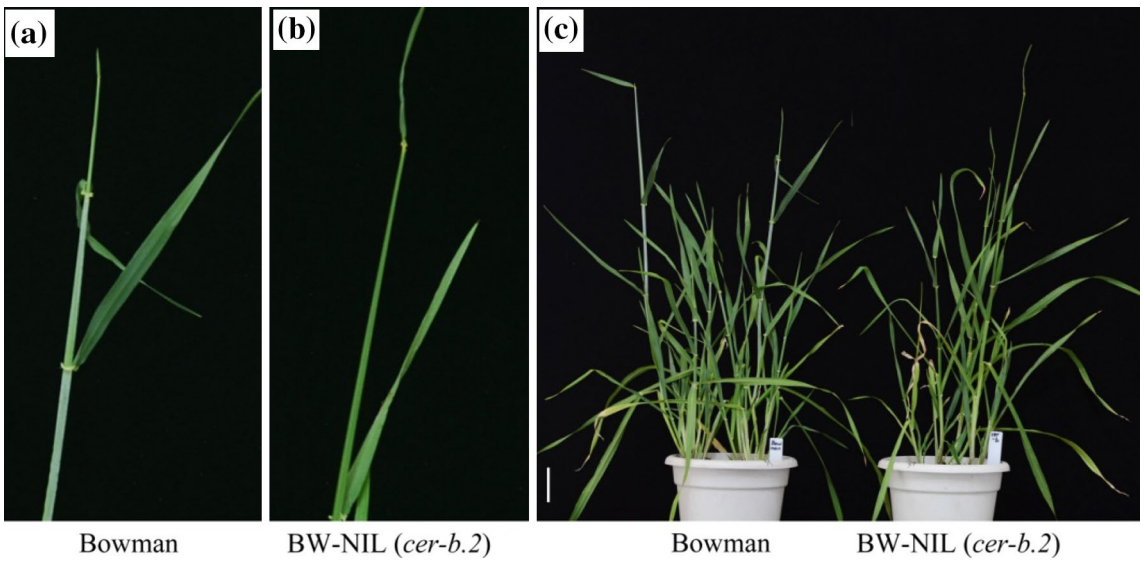

Fig. 1 The flag leaf sheaths of **a** cv. Bowman, **b** BW-NIL (*cer-b.2*). **c** The whole plant appearance of BW-NIL (*cer-b.2*) and wild type cv. Bowman. *Scale bar* 10 cm

The mutant's β-diketone deficiency is compensated by an enhanced content of primary alcohols, aldehydes, fatty acids and alkanes

The amount of cuticular wax deposited on the BW-NIL (*cer-b.2*) plant was less than that on cv.Bowman (Table 2). GCMS analysis demonstrated that 14,16-hentriacontanedione, an abundant component of the leaf sheath wax produced by cv. Bowman (Fig. 3a), was absent in the wax produced by BW-NIL (*cer-b.2*) (Fig. 3b; Table 2). Instead, the BW-NIL (*cer-b.2*) leaf sheath wax contained more very long chain alcohols (72% more than in cv. Bowman) and, to a lesser extent, more very long chain aldehydes (12%) (Table 2). The content of C_{26} primary alcohol was significantly greater in the mutant than in the wild type (Figs. 3, 4a), as were those of C_{26} aldehydes, C_{26} fatty acids, and C_{25} and C_{29} alkanes (Fig. 4). The deficiency of β-diketone in the mutant, which led to significant changes in the total waxes between mutant and wild type (Table 2), was partially offset by an increased content of other aliphatics.

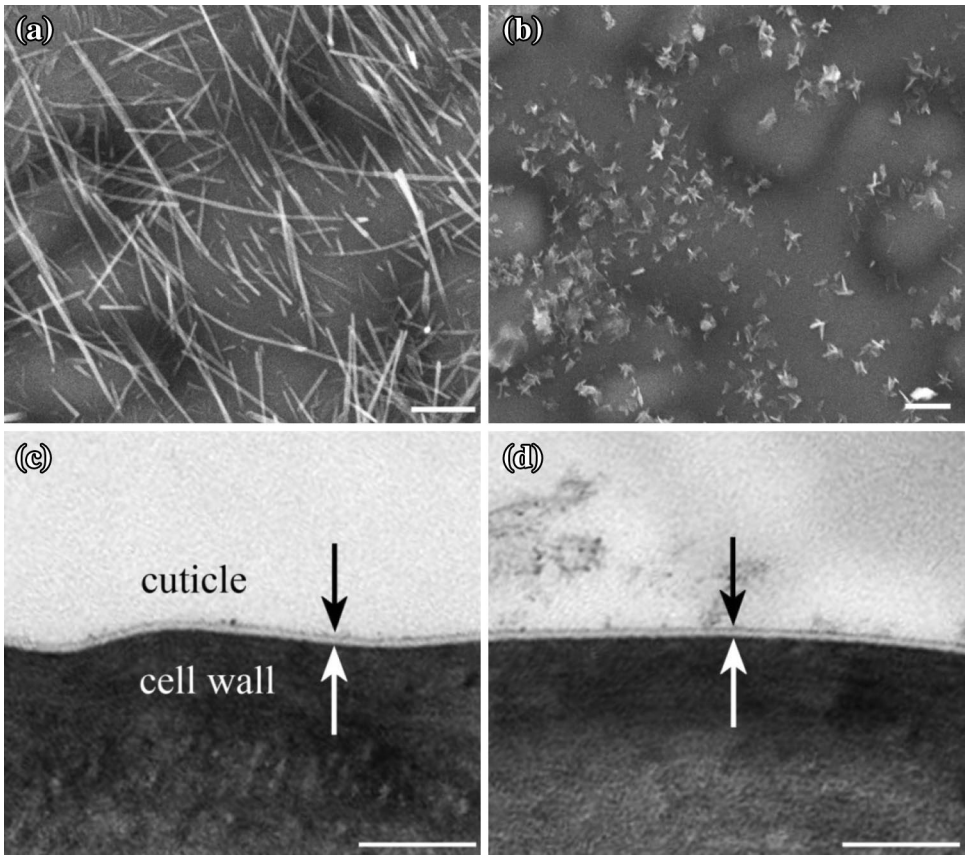

Fig. 2 SEM (**a, b**) and TEM (**c, d**) imaging of the adaxial surface/cuticle of leaf sheaths of **a, c** cv. Bowman, **b, d** BW-NIL (*cer-b.2*). The *arrows* in **c, d** indicate the thickness of cuticle. *Scale bars* **a, b** 3.0 µm, **c, d** 0.25 µm

Table 2 The wax composition of the leaf sheath of cv. Bowman and the BW-NIL (*cer-b.2*) mutant

Compound class	Measurement	Bowman	BW-NIL (*cer-b.2*)	Fold change
Primary alcohols	Amount	0.92 ± 0.22	5.52 ± 1.66**	6.0
	Percentage	6.28 ± 1.53	77.86 ± 22.3	
Fatty acids	Amount	0.34 ± 0.06	0.43 ± 0.13	1.26
	Percentage	2.34 ± 0.40	6.06 ± 1.68	
Aldehydes	Amount	0.05 ± 0.03	0.89 ± 0.45*	17.8
	Percentage	0.36 ± 0.23	12.55 ± 6.03	
Alkanes	Amount	0.15 ± 0.06	0.25 ± 0.09	1.6
	Percentage	1.02 ± 0.42	3.53 ± 1.20	
14,16-Hentriacontanedione	Amount	8.57 ± 1.31	0.00 ± 0.00***	0
	Percentage	58.58 ± 8.95	0.00 ± 0.00	
UN	Amount	4.60 ± 1.86	0.00 ± 0.00**	0
	Percent	31.42 ± 12.74	0.00 ± 0.00	
Total	Amount	14.64 ± 3.23	7.09 ± 1.80**	0.51

Mean total wax amount (µg/cm^2 ± SD), total amount of each wax group (µg/cm^2 ± SD), and percentage of each group within each sample extract are shown

UN unknown compounds

Level of significance obtained with a Student's *t* test are marked by following: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Linkage mapping of the gene mutated in BW-NIL (*cer-b.2*)

The 23–19 × BW-NIL (*cer-b.2*) mutant F_1 plants were glaucous, confirming that the mutation acted recessively. The ratio of glaucous to glossy F_2 progeny was 694:256, which was consistent with a 3:1 ratio ($\chi^2 = 1.84$, $p = 0.16$), thereby supporting the notion that the mutation involved a single gene. The candidate chromosome 3HL region harboring the gene is flanked by the markers 2_1513

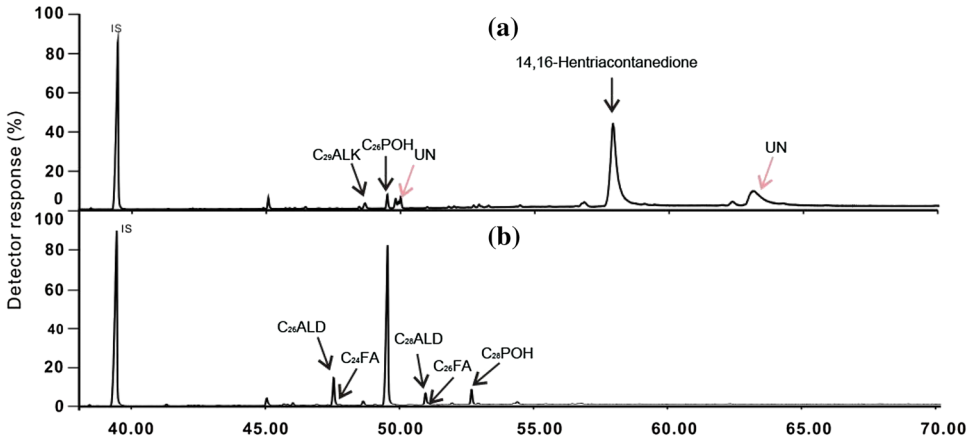

Fig. 3 Representative GCMS chromatograms of extracts of the leaf sheath of **a** cv. Bowman, **b** BW-NIL (*cer-b.2*). The *vertical axis* shows relative abundance. *IS* internal standard (*n*-tetracosane). The *black arrows* indicate primary alcohols (POH), fatty acids (FA), aldehydes (ALD), *n*-alkanes (ALK) and 14,16-hentriacontanedione; the *orange arrows* indicate unidentified compounds (UN)

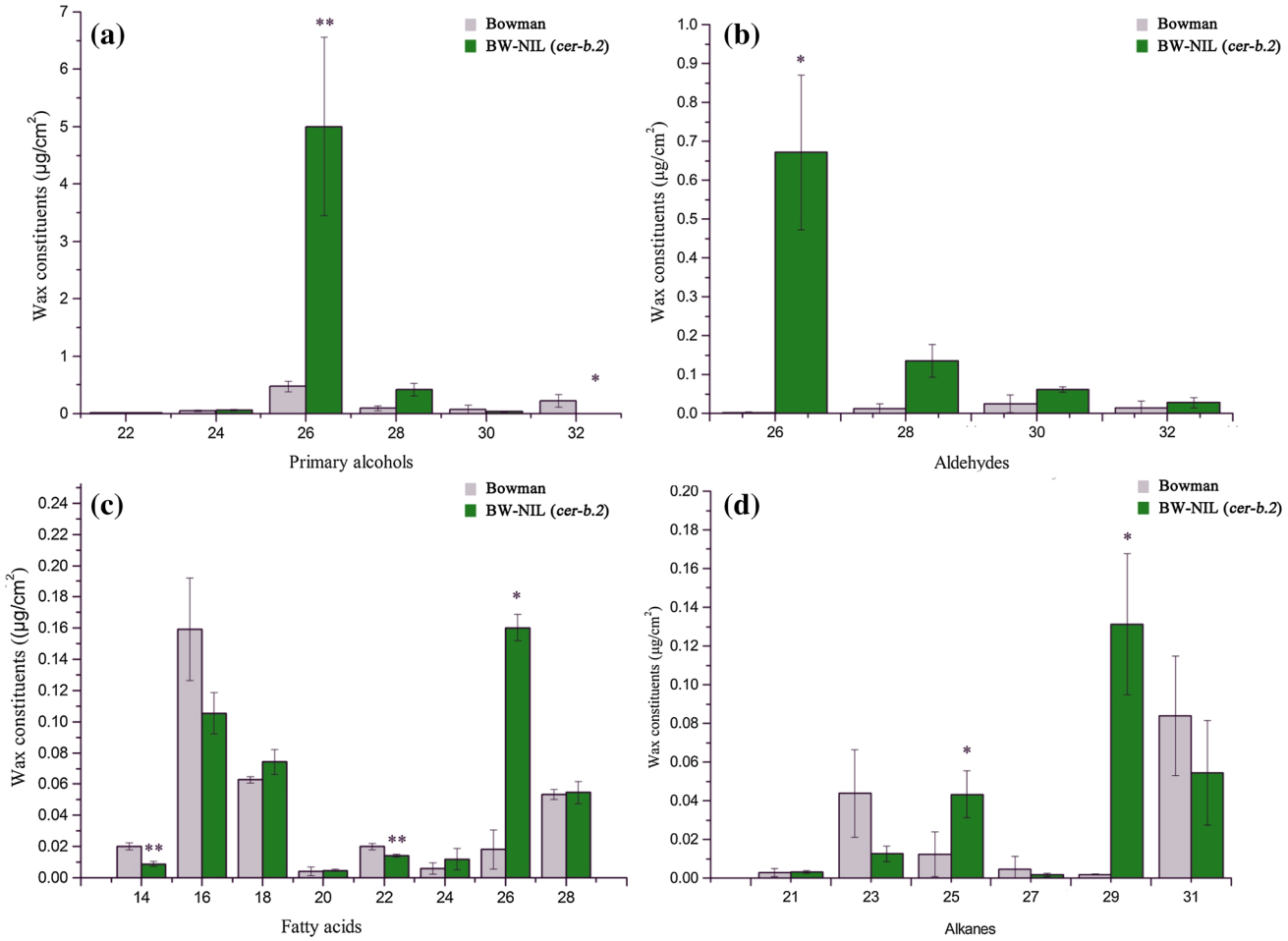

Fig. 4 GCMS-based quantification of the constituents of the cuticular wax formed on the leaf sheaths of cv. Bowman (shown in *grey*) and BW-NIL (*cer-b.2*) (shown in *green*) plants. The components included **a** primary alcohols, **b** aldehydes, **c** fatty acids and **d** alkanes. The *whiskers* indicate the standard error ($n=3$). Means differed significantly from one another at *, **$p<0.05$, <0.01. (Color figure online)

and 1_0754 (Druka et al. 2011; Fig. 5a). The genes lying within this region defined by 2_0626 and 3_0137 in the barley genome zipper (Mayer et al. 2011) were used as search queries against the BW-NIL (*cer-b.2*) and 23–19 unigenes. Fifteen genes, polymorphic between the parental lines, were identified: of these, nine (MLOC_5326,

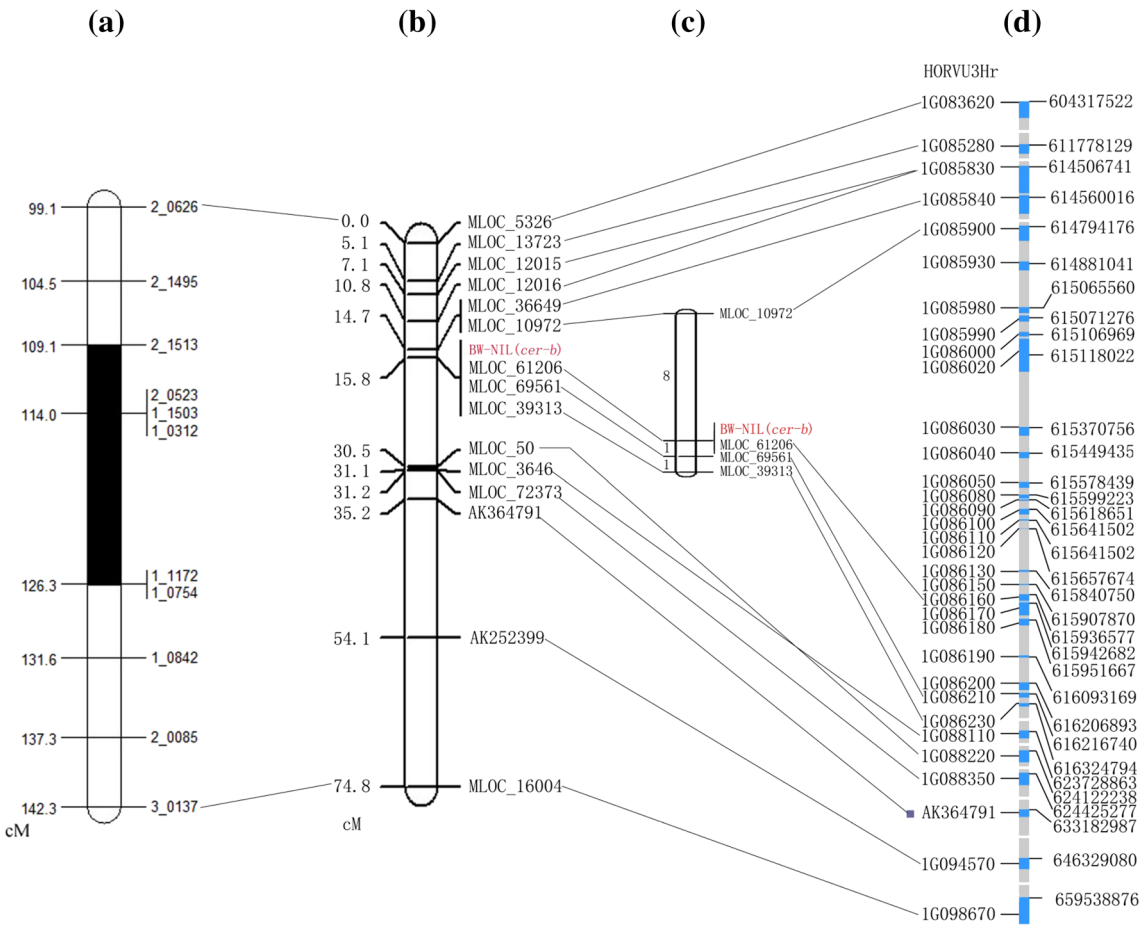

Fig. 5 A localized genetic map of barley chromosome arm 3HL, the site of the gene underlying the *cer-b.2* mutant. **a** Introgression region in barley near isogenic lines, BW-NIL (*cer-b.2*), map positions follow by Druka et al. (2011). **b** Linkage map based on 93 F_2 progeny of the cross 23–19×BW-NIL (*cer-b.2*) selected to express the mutant phenotype, **c** a higher resolution map based on 708 gametes in the same F_2 population. The numbers shown on the left side of the map indicate the number of recombinants recovered, **d** the physical map of the region harboring the *cer-b* gene (International Barley Genome Sequencing Consortium 2016). The purple square shows the gene *AK364791* which sequence could be found on chromosome 3H, but is not annotated

_13723, _12016, _69561, _3646, _72373, _16004 and AK364791, AK252399) were used to genotype 93 of the 23–19×BW-NIL (*cer-b.2*) F_2 progeny with the mutant phenotype. MLOC_69561 was completely linked to *cer-b*. The sequences of the 58 genes surrounding MLOC_69561 obtained from the barley physical map (International Barley Genome Sequencing Consortium 2012) were then each used in turn as a BlastN search query against the 23–19 and BW-NIL (*cer-b.2*) unigene sets. Four SNP containing genes (MLOC_12015, MLOC_61206, MLOC_10972 and MLOC_36649) and one containing an indel (MLOC_50) were detected. The assays for these polymorphisms were subsequently applied to the mapping population. In addition, the gene MLOC_39313 was found by genomic DNA resequencing to harbor a SNP in its intronic sequence, so could also be mapped. The mapping (Fig. 5b; associated markers listed in Table 1) led to the conclusion that the mutant gene was flanked by MLOC_10972 and _50 and co-segregated with MLOC_61206, _69561, and _39313. To improve the level of mapping resolution, an additional 261 F_2 progeny were then genotyped using the key markers. Based on the 708 ($93\times2+261\times2$) gametes, eight recombinants between MLOC_10972 and _61206 were recovered, one between MLOC_61206 and _69561, one between MLOC_69561 and _39313 and none between *cer-b* and MLOC_61206 (Fig. 5c). As a result, the location of the mutated gene was narrowed to within the ~1.3 cM interval flanked by MLOC_10972 and _69561. According to the current version of the barley genome sequence (International Barley Genome Sequencing Consortium 2016), this interval houses 20 genes (Fig. 5d; Supplemental Data Set 1). Out of the 20 genes, only *HORVU3Hr1G086000* gene, encoding a Myb family

transcription factor protein, is likely related to cuticular wax formation (Yeats and Rose 2013). The identification of the mutation in this gene in *cer-b* mutants awaits further study.

Discussion

A block in the β-diketone synthesis pathway is responsible for the glossy leaf sheath of BW-NIL (*cer-b.2*)

BW-NIL (*cer-b.2*) plants formed non-glaucous leaf sheaths and their epicuticular wax across the entire leaf sheath surface formed platelets rather than tubules. The wax was completely depleted for 14,16-hentriacontanedione, which was compensated for by a higher content of C_{26} primary alcohols and other very long chain aliphatics. Waxes present on glaucous barley spikes and stems in the form of long tubules appear to have a high β-diketone content (von Wettstein-Knowles 1972). Like BW-NIL (*cer-b.2*), the *cer-c* and *cer-q* mutants also produce glossy leaf sheaths and spikes, and are compromised with respect to β-diketone synthesis (von Wettstein-Knowles 1972). The relationship between glaucousness and the presence of β-diketone components also obtains in wheat (Adamski et al. 2013; Bianchi and Figini 1986; Tsunewaki and Ebana 1999; Varshney et al. 2006). The cuticular wax present on the wheat leaf sheath, peduncle and spike is dominated by β-diketones, whereas the wax present on the leaf blade has a high primary alcohol content; the former wax tends to form tubules, while the latter forms platelets (Wang et al. 2015). The indication is thus that the change from tubular to platelet wax is a consequence of 14,16-hentriacontanedione being replaced by very long chain aliphatics in *cer-b* mutant. However, the amount of unknown compounds (4.6 μg cm^{-2}), representing about one-third of the total cuticular wax of wild type, was also absent in the mutant. The product of *Cer-b* may play a role in the synthesis of these compounds, which in turn could contribute to the mutant phenotype. As such, further studies are required to identify the unknown compounds.

Cer-b is a new gene involved in β-diketone synthesis

The *cer-b.2* mutant resembles *cer-c*, *-q*, and -u with respect to its effect on β-diketone content. Genetic and biochemical investigations have shown that the genes underlying the *cer-c, -q* and *-u* mutants are clustered together, each encoding a key component in the β-diketone synthase polyketide pathway in which FAE enzyme complexes and type III PKSs are important (Schneider et al. 2016; von Wettstein-Knowles 2012). The PKSs promote fatty acid elongation to yield β-diketone by leave out one or more of the three reactions removing the β-keto group in given elongation cycles by KCR (β-ketoacyl-CoA ketoreductase), HCD (β-hydroxyacyl-CoA dehydratase), ECR (β-enoyl-CoA reductase) (Hen-Avivi et al. 2016; Schneider et al. 2016). The *cer-c* mutation affects a gene encoding chalcone synthase, while the *Cer-q* gene encodes a lipase/carboxyl transferase and the *Cer-u* gene a P450 enzyme (Schneider et al. 2016). The *Cer-cqu* cluster maps to a sub-telomeric region on the short arm of chromosome 2H (Schondelmaier et al. 1993; Tsuchiya 1972), which is quite distinct from the location of the *cer-b.2* gene. The inference is that the *Cer-b* mutation is unrelated to the *cer-cqu* cluster.

The *cer-b* mutation is also unrelated to the β-diketone metabolic genes in the wheat *W1* cluster because almost all of the genes within W1 are represented in the *Cer-cqu* cluster (Hen-Avivi et al. 2016). *Cer-b* might be a gene encoding a transcription factor that regulates the biosynthesis of β-diketones. The plant metabolic gene clusters appear not to harbor genes regulating the corresponding pathway. In an example, GLYCOALKALOID METABOLISM9, an AP2 transcription factor encoded by a gene located on tomato chromosome 1, could likely regulate a cluster of steroidal glycoalkaloid (SGA) pathway genes on chromosome 7 (Cárdenas et al. 2016). Out of the 20 candidate genes in the *cer-b.2* interval, *HORVU3Hr1G086000*, a gene encoding a MYB family transcription factor, is likely related to cuticular wax formation. R2R3-type MYB16 and MYB106 are required for cuticle development in *Arabidopsis* and *Torenia fournieri* (Oshima et al. 2013). MYB94 and MYB96 additively activate cuticular wax biosynthesis in *Arabidopsis* (Lee et al. 2016). Cer-b.2 might be the MYB protein HORVU3Hr1G086000 regulating the β-diketone metabolic genes in the *Cer-cqu* cluster on chromosome 2H. A search of the InterPro (http://www.ebi.ac.uk/interpro/) database with the HORVU3Hr1G086000 sequence identified a domain (designated IPR017930) which recognizes the sequence YAAC(G/T)G. When PlantCARE software (bioinformatics.psb.ugent.be/webtools/plantcare/html/) was applied to identify the likely HORVU3Hr1G086000 binding site, it highlighted the sequences CAACTG (present in the *Cer-cqu* promoters) and TAACTG (in the *Cer-u* promoter) (Table S1; Table 2). This suggests that the target of HORVU3Hr1G086000 may be the β-diketone metabolism genes harbored by the *Cer-cqu* cluster on chromosome 2H. In wheat, by comparison, MYB31 and MYB74 act to activate cuticle synthesis by regulating the genes encoding a cytochrome P450 mono-oxygenase (ATT1) and a 3-ketoacyl CoA synthetase (KCS1) (Bi et al. 2016). As yet, none of the other 19 candidate genes present in the *cer-b.2* segment has been connected with the formation of the cuticle, but the possibility that one of these is equivalent to the *Cer-b* gene cannot yet be ruled out. A simple way of reducing

the number of candidate genes to be considered will be to adopt a fine-mapping approach.

Author contribution statement GC and TK conceived the ideas and designed the investigation; QZ, CL, and GC conducted the experiments, analyzed data and drafted the manuscript. XYM, RJD and ADW assisted the experiments. JWZ and JCZ collected samples. KM and GC did RNA-seq analysis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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