A Metabolic Gene Cluster in the Wheat \textit{W1} and the Barley \textit{Cer-cqu} Loci Determines $\beta$-Diketone Biosynthesis and Glaucoyness

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Metabolic Gene Cluster for β-Diketone Biosynthesis

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Synopsis
A metabolic gene cluster underlying the known glaucousness loci W1 in wheat and Cer-cqu in barley establishes a novel molecular pathway for β-diketone wax biosynthesis

Abstract
The glaucous appearance of wheat and barley plants, that is the light bluish-grey look of flag leaf, stem, and spike surfaces, results from deposition of cuticular β-diketone wax on their surfaces; this phenotype is associated with high yield, especially under drought conditions. Despite extensive genetic and biochemical characterization, the molecular genetic basis underlying the biosynthesis of β-diketones remains unclear. Here we discovered that the wheat W1 locus contains a metabolic gene cluster mediating β-diketone biosynthesis. The cluster comprises genes encoding proteins of several families including type-III polyketide synthases, hydrolases, and cytochrome P450s related to known fatty acid hydroxylases. The cluster region was identified in both genetic and physical maps of glaucous and glossy tetraploid wheat, demonstrating entirely different haplotypes in these accessions. Complementary evidence obtained through gene silencing in planta and heterologous expression in bacteria supports a model for a β-diketone biosynthesis pathway involving members of these three protein families. Mutations in homologous genes were identified in the barley eceriferum mutants defective in β-diketone biosynthesis, demonstrating a gene cluster also in the β-diketone biosynthesis Cer-cqu locus in barley. Hence, our findings open new
opportunities to breed major cereal crops for surface features that impact yield and stress response.

INTRODUCTION

The extracellular matrix glazing aerial plant organs, the cuticle, serves as an interface with the environment and thus strongly contributes to fitness in unceasingly varying growth conditions. The lipophilic cuticular layer and epicuticular depositions contain complex lipid mixtures ‘tailored’ towards specific roles in development, stress responses, and interactions with other organisms, and thus varying greatly between species, organs, and growth stages. The polyester cutin, a matrix of cross-linked hydroxy C$_{16}$ and/or C$_{18}$ fatty acids, forms a major constituent of the cuticle (Yeats and Rose, 2013). The cutin scaffold holds the cuticular waxes, typically complex mixtures of very-long-chain fatty-acid (VLCFA) (C$_{20}$–C$_{34}$) derivatives (Samuels et al., 2008; Yeats and Rose, 2013). The wax compounds common to all plants are formed in three major stages within epidermal cells, starting with the synthesis of C$_{16}$ fatty acid in plastids, then by further elongation to C$_{20} – C_{34}$ fatty acids in the endoplasmic reticulum (ER), and finally modification either along a wax alkane-forming pathway or an alcohol-forming pathway (Supplemental Figure 1). Plastidial elongation to C$_{16}$ depends on the fatty acid synthase (FAS) systems catalyzing four reactions, initially forming a β-ketoacyl-ACP intermediate and then involving the step-wise conversion of the keto function into CH$_{2}$. Similarly, the further elongation to VLCFAs involves fatty acid elongase (FAE) systems carrying out analogous reactions, albeit with acyl-CoA intermediates.

In the last decade, major discoveries have greatly advanced our genetic and biochemical understanding of cuticle biosynthesis and assembly, the spatial and temporal transcriptional control of associated metabolic pathways and, to some extent, structure-function relationships (Yeats and Rose, 2013; Borisjuk et al., 2014; Hen-Avivi et al., 2014). Nevertheless, as most of the work on cuticle biology has been carried out in model plant species such as Arabidopsis thaliana, maize (Zea mays), tomato (Solanum lycopersicum), and rice (Oryza sativa), the unique surface features of many other species or families remain poorly understood. In particular, many Poaceae species (including wheat [Triticum spp.], barley [Hordeum vulgare], rye [Secale cereale],
and oat [*Avena sativa*]) produce very-long-chain $\beta$-diketones with characteristic structures likely formed along pathways different from those leading to more ubiquitous wax compounds (Supplemental Figure 1). Despite many years of research on $\beta$-diketone biosynthesis, particularly in barley and wheat (von Wettstein-Knowles, 1976; von Wettstein-Knowles and Søgaard, 1980; von Wettstein-Knowles, 1995; Adamski et al., 2013; Zhang et al., 2013; Zhang et al., 2015), the genes and enzymes involved remain to be discovered.

Glaucousness, the light bluish-grey appearance of flag leaf, stem, and spike surfaces due to wax crystals deposited on the surfaces is a morphological trait that varies genetically between different plant species. The opposite form is known as glossy (gl), non-glaucous, eceriferum (cer), bloomless (bm), or waxless. Interestingly, the vast majority of the modern wheat (*Triticum aestivum* L.) cultivars exhibit a glaucous appearance due to the presence of $\beta$-diketones, while both glaucous and glossy phenotypes are found in wild wheat species. Early genetic studies revealed that this trait in hexaploid wheat is predominantly governed by four loci, namely the wax production loci $W1$ and $W2$ and the wax inhibition loci $Iw1$ and $Iw2$. $W1$ and $Iw1$ reside on the short arm of chromosome 2B (2BS), whereas $W2$ and $Iw2$ are located on 2DS (Tsunewaki, 1962; Tsunewaki and Ebana, 1999). Either one of the wax production loci, $W1$ or $W2$, is sufficient to produce a glaucous phenotype. The wax inhibitor loci have a dominant epistatic interaction with the wax production loci, and the presence of either $Iw1$ or $Iw2$ (or both) suppresses the glaucous phenotype.

Barley is a diploid cereal that produces $\beta$-diketones as wax constituents and can therefore serve as an excellent complementary study system together with wheat. Previous genetic work in barley demonstrated that the $\beta$-diketone biosynthesis-associated *Cer-cqu* locus resides in a sub-telomeric region on the short arm of chromosome 2H (2HS) (Takahashi et al., 1953; Tsuchiya, 1972; Schondelmaier, 1993), in a syntenic position to the wheat *W1* locus (Tsunewaki and Ebana, 1999; Kole et al., 2006). Extensive genetic and biochemical studies in barley proposed a putative $\beta$-diketone biosynthesis pathway, which includes three enzymatic steps (von Wettstein-Knowles, 1995; von Wettstein-Knowles, 2012). Two of these were hypothesized to be catalyzed by type-III polyketide synthase (PKS) enzymes, Cer-c and Cer-q. The third
step, $\beta$-diketone hydroxylation, was predicted to be catalyzed by Cer-u. It remained unclear whether $\beta$-diketone biosynthesis in barley is controlled by proteins encoded by three separate but very tightly linked genes ($Cer-c$, $q$, and $u$) or by a single, multifunctional protein with at least three domains ($c$, $q$, and $u$) encoded by a single $Cer-cqu$ gene. The latter scenario was favored (von Wettstein-Knowles and Søgaard, 1981; von Wettstein-Knowles, 1995; von Wettstein-Knowles, 2012). Very recently, Schneider et al. (2016) suggested that three different genes rather than one encoding a multifunctional enzyme are involved in the $\beta$-diketone biosynthesis pathway in barley.

In the present work, we discovered a metabolic gene cluster in wheat and in barley comprising genes that are responsible for $\beta$-diketone biosynthesis and encode a PKS, a cytochrome P450 and, unexpectedly, a hydrolase/carboxylesterase. The identification and characterization of the $\beta$-diketone biosynthetic route as well as additional results reported here will promote innovative breeding schemes that target plant surface characteristics in major cereal crops.
RESULTS

Analysis of the glaucousness trait using chromosome-arm substitution lines of wild emmer in common wheat

To study β-diketone waxes in wheat, we used a population of lines described by Millet et al. (2013), in which a series of chromosome-arm substitution lines (CASLs) of wild emmer *Triticum turgidum* ssp. *dicoccoides* accession TTD140 (2n = 4x = 28; genome AABB) were produced in the background of the glaucous common bread wheat *T. aestivum* cultivar Bethlehem (BL) (2n = 6x = 42; genome AABBDD). One of the most notable observations was the glossy phenotype of CASL*2BS* (the short arm of chromosome 2B of wild emmer in the background of common wheat), whereas all the other CASLs had a glaucous appearance. The parental genotype BL and the CASL*2BS* were therefore selected to study cuticular wax biosynthesis and composition (for an illustration of lines see Supplemental Figure 2). The glaucous phenotype of BL (therefore *iw1/iw1*) and the glossy phenotype of CASL*2BS* [carrying *Iw1/Iw1* alleles of TTD140 (Rong et al., 2000)] were noticeably visible in flag leaves, peduncles, leaf sheaths, and spikes (Figure 1 A). Cryo-scanning electron microscopy (cryo-SEM) revealed differences in the relative amounts and shape of epicuticular wax crystals between these genotypes (Figure 1B). While the flag leaves and peduncles of BL were covered with tubule-shaped crystals typical for β-diketone-rich wax (Bianchi and Figini, 1986; Riederer M, 2006), the CASL*2BS* tissues accumulated platelet-shaped crystals characteristic of alcohol-rich wax (Riederer M, 2006) (Figure 1B). The *W1* and *Iw1* loci are known to play a role in wax biosynthesis and to be located on 2BS, suggesting that these two loci are the base of the different wax phenotypes.

Surface wax analysis reveals that the glossy CASL*2BS* plants lack β-diketone, its hydroxy-β-diketone derivatives and related 2-alkanol esters

To determine the underlying reason for the visible differences in glaucousness between BL and the CASL*2BS*, flag leaf blades and flag leaf sheaths plus peduncles of flowering plants were harvested and their cuticular waxes analyzed using Gas Chromatography (GC) with Mass Spectrometry (MS) and Flame Ionization Detection (FID). CASL*2BS* had significantly less total wax, reduced by 20% and 80% in flag leaf
blade and flag leaf sheath/peduncle samples, respectively, compared to the equivalent wild-type BL tissues. Wax composition also differed between the genotypes, with β-diketone and hydroxy-β-diketones together making up 13% and 79% of total wax amounts in BL flag leaf blades and sheaths/ peduncles, respectively, whilst completely absent in the CASL*2BS (Figure 1C, D). In addition, only traces of 2-alkanol esters were detected in both CASL*2BS tissues, and free fatty acids were severely depleted in the CASL*2BS leaf sheaths. Conversely, alcohols, esters and aldehydes accumulated to higher levels in leaf sheaths and peduncle of CASL*2BS, whereas these were low in these tissues of BL. In particular, the marked difference in β-diketone accumulation between the genotypes is in agreement with the accepted impact of β-diketones on glaucousness (Barber and Netting, 1968; Bianchi and Figini, 1986; Adamski et al., 2013; Zhang et al., 2013).

Transcriptome analysis implicates three genes in β-diketone production in hexaploid bread wheat

In order to decipher the β-diketone biosynthesis pathway, transcriptome analysis was conducted in which we compared gene expression in peduncle tissues derived from the BL and CASL*2BS genotypes. Filtering for at least 2-fold changes in expression levels between the two genotypes revealed 628 and 316 transcripts that were more highly expressed in BL or CASL*2BS, respectively. Examination of annotations of these differentially expressed transcripts revealed that one of the genes most highly expressed in BL encodes a putative member of the type-III polyketide synthase (PKS) enzyme family, with significant sequence similarity to curcuminoid synthase in turmeric, *Curcuma longa* (Ramirez-Ahumada et al., 2006). One of the best-known plant PKSs is chalcone synthase (CHS), which catalyzes the formation of naringenin chalcone in the flavonoid biosynthesis pathway (Ferrer et al., 1999; Jez et al., 2000). Interestingly, querying the *PKS-like* transcript against the available *T. aestivum* genome ((IWGSC), 2014) database revealed the presence of matching genomic sequences on 2BS and 2DS, the chromosomal arms known to carry the *W1* and *W2* loci, respectively (Tsunewaki and Ebana, 1999) (Table 1). Together with the evidence from previous biochemical studies in barley, which implicated type-III PKSs (termed pkKCS) in β-
diketone formation (von Wettstein-Knowles, 2012), this PKS-like transcript was a prime candidate to be involved in wheat β-diketone biosynthesis.

Out of 628 transcripts highly expressed in BL, 139 mapped to chromosome arm 2BS, and two were identified beside the PKS as strong candidates to be associated with β-diketone biosynthesis. The first gene was annotated as a putative carboxylesterase or hydrolase. Carboxylesterases could catalyze the hydrolysis of long-chain fatty acid esters and thioesters, including CoA or acyl carrier protein (ACP) esters. The second candidate gene encoded a putative cytochrome P450 protein with similarity to CYP709C1. Members of the 709C1 subfamily of cytochrome P450s have been previously associated with sub-terminal hydroxylation of long-chain fatty acids, an enzymatic step that is also thought to be required for the formation of hydroxy-β-diketones. This candidate was annotated as CYP709J4 (see Materials and Methods). In barley, β-diketone biosynthesis genes were reported to be located on chromosome arm 2HS (Takahashi et al., 1953; Tsuchiya, 1972; Schondelmaier, 1993). Consistent with this, all three wheat candidate genes had homologous sequences on barley 2HS (Table 1). The results thus identified these three genes as strong candidates for the W1 locus, and we therefore decided to subject them to further characterization. They were designated as Diketone Metabolism-PKS (DMP), -Hydrolase (DMH) and -CYP450 (DMC) (Table 1, for accession numbers see Materials and Methods).

Molecular characterization of chromosome arm 2BS demonstrates that the three candidate genes are not found in lines TTD140 and CASL*2BS

The glaucous phenotype of BL must be an iw1iw2 genotype combined with W1 and/or W2 loci, while CASL*2BS carries the TTD140 lw1 (Millet et al., 2013) and W1 with unknown genotype instead. The W2 genotype of BL and CASL*2BS was not determined in this study; however, BL and CASL*2BS have the same W2 locus. Therefore, BL and CASL*2BS differ in the lw1 loci and possibly but not necessarily in their W1 loci. To determine genotypic differences between BL and CASL*2BS, both lines were genotyped with the 90K iSelect SNP Chip (Wang et al., 2014b). This revealed 422 markers that were polymorphic between the genotypes, of which 12 showed no amplification for CASL*2BS but were detected in the BL (referred to as null markers; Supplemental Table 1). Further 90K chip genotyping revealed that the same 12
polymorphic markers were also nulls in the glossy wild emmer wheat *T. turgidum* ssp. *dicoccoides* accession TTD140 (the 2BS donor in CASL*2BS), but present in the glaucous durum wheat (*T. durum*) 2n = 4x = 28; genome AABB) cultivar Svevo.

The 422 polymorphic markers were ordered along the chromosome arm 2BS of wheat using available genetic maps (Avni et al., 2014; Wang et al., 2014b; Maccaferri et al., 2015) as references. The mapping revealed that the 12 null SNP markers are genetically linked and absent in the glossy genotypes. Importantly, four of these 12 closely linked markers were found to reside inside the *DMC* gene and another three inside the *DMP* gene (Supplemental Table 1). The third candidate gene, *DMH*, is not represented on the 90K iSelect genotyping chip.

To confirm our genotyping results, we attempted to amplify the three candidate genes with specific oligonucleotide pairs from the tetraploid glaucous cultivar Svevo and the glossy TTD140 genotype. While the expected fragments could be amplified from Svevo, no amplification products were observed for TTD140, hence confirming the absence of *DMP*, *DMH*, and *DMC* in TTD140 (Supplemental Figure 3).

**The fine map of 2BS in tetraploid wheat reveals complete genetic linkage between the three β-diketone metabolism candidate genes, *W1* and the *Inhibitor of W1* (*Iw1*) loci**

Fine genetic mapping of the three candidate genes was performed using segregating populations based on crosses between glaucous and glossy genotypes. One segregating population was developed using the tetraploid accessions Kofa+*Lr19* (glaucous) and AUS2499 (glossy). AUS2499 is a mutant derived from a glaucous line. The F₁ hybrids were glaucous, confirming that *Iw1* is not present in AUS2499 or in Kofa+*Lr19*. Analysis of 352 F₂ progeny revealed the expected 3:1 segregation ratio for a single locus, which was mapped to 2BS. Examination of another 4,423 F₂ progeny suggested that the dominant locus for β-diketone production designated *W1* is confined to a 0.16 cM interval flanked by markers *W1_3* and *W1_4* (Figure 2 A, Supplemental Text 1, Supplemental Table 2).

A second segregating population developed between Svevo (glaucous) and TTD140 (glossy) was subsequently examined. Phenotypic evaluation of F₁ hybrids displayed a clear glossy phenotype, supporting previous information regarding the
dominant inhibitor (Iw1) on chromosome arm 2BS in TTD140 (Rong et al., 2000).
Examination of 299 F₂ progeny revealed a single locus with 1:3 (glaucous: glossy)
Mendelian segregation ratio. The single locus (corresponding to Iw1) was mapped to
2BS, completely linked to JIC0009. The markers Xgwm614 and JIC0010 were mapped
in a distance of 0.84 cM (distal) and 0.33 cM (proximal) to JIC009, respectively. We next
genotyped the F₂ population with the null markers for DMP, DMH, and DMC, and found
a complete linkage to JIC0009. Taken together, both W1 and Iw1 are located in very
close proximity to JIC0009 and the three candidate genes (Figure 2B, Supplemental
Text 1, Supplemental Table 2).

The three candidate β-diketone biosynthesis genes are part of a metabolic gene
cluster in the genome of glaucous wild emmer wheat accession Zavitan and are
missing in glossy wild emmer wheat accession TTD140
Analysis of the W1/Iw1 interval (between genetic markers JIC036 and JIC032, see
Figure 2C) in the recently sequenced genome of the glaucous wild emmer wheat
accession Zavitan resulted in 22 predicted genes (including partial genes and
pseudogenes) (Please see accession numbers in Material and Methods). Six genes
encode members of the CYP450 family (P450), five genes encode members of the
polyketide synthase (PKS) family and another four encode members of the
esterase/lipase family (Hydrolase; HYD), thus accounting for 15 of the 22 genes (Figure
2C). This analysis showed that the three candidate genes are not only located on 2BS
but are also tightly clustered together in the W1 wheat locus. The high sequence
similarity among the duplicated genes (>99.3%) together with their physical
arrangement suggested that a series of independent local duplications have occurred
within the Zavitan interval after the wheat-barley divergence (Supplemental Text 2,
Supplemental Table 3, Supplemental Figure 4). A phylogenetic tree of the six P450
proteins from Zavitan with other related P450s showed that these proteins are
associated with two similar but distinct P450 clades, namely that P450-1.1, P450-1.2
(annotated as CYP709J4 orthologs, see Materials and Methods) and P450-2.1, P450-2.2
(annotated as CYP709J5, partial) are similar to wheat CYP709C1, and that a third
pair comprising P450-3.1 and P450-3.2 (annotated as CYP96B30) is related to
Arabidopsis MAH1 (CYP96A15) (Figure 2D). Both wheat CYP709C1 and Arabidopsis
MAH1 catalyze in-chain hydroxylation (Kandel et al., 2005; Greer et al., 2007), and many of the P450s from related clades are known to be involved in fatty acid metabolism (Figure 2D). Surprisingly, in addition to the 15 DMP/H/C homologs, a WES (wax ester synthase) belonging to the membrane-bound O-acyl transferase family (MBOAT) resides in this region (Figure 2C). The β-diketone biosynthesis pathway branch out to 2-alkanol esters (Supplemental Figure 1), therefore it is likely that the WES gene might be also associated with the β-diketone biosynthesis gene cluster.

Additionally, BAC sequences of the wild emmer TTD140 were employed to analyse the candidate gene regions in a glossy wheat line. Genetic markers JIC007/IWB7407 (distal) and JIC010/CD927782 (proximal) flanking the W1/Iw1 interval were anchored (Figure 2C). While the distal and proximal regions of the interval share ≥96% similarity, the interval between is completely different between TTD140 and Zavitan (over 800 kb in the TTD140 sequence). This is true for both the intergenic space as well as for the gene content, with the exception of two NB-Arc-like genes (NB-Arc-like_1 and _2; Figure 2C, Supplemental Text 3), pointing to haplotype divergence across the W1/Iw1 interval between these two wild emmer accessions.

The candidate β-diketone biosynthesis genes are tightly co-expressed, and their expression corresponds to the β-diketone accumulation pattern

If DMP, DMH, and DMC encode enzymes involved in β-diketone biosynthesis, their expression is expected to correlate with β-diketone accumulation. In glaucous tissues, β-diketones start accumulating towards plant maturation at the uppermost internode, approximately two weeks prior to ear emergence. By the time the ear has fully emerged, the flag leaf and the stem become visually glaucous (Troughton and Hall, 1967). Expression of the three candidate genes was examined in BL lower versus upper internodes at a time when the glaucous phenotype becomes visible (Figure 3A). All three genes displayed significantly higher expression in the upper, glaucous internode than in the lower, glossy ones (Figure 3B-D). Moreover, these genes exhibited highly significant co-expression between each other (r > 0.85) (Figure 3E and Supplemental Data Set 1), further supporting their involvement in wheat β-diketone production.
Silencing of DMP and DMH provides in planta evidence for their role in the wheat β-diketone biosynthetic pathway

The DMP and DMH genes were silenced using Barley stripe mosaic virus (BSMV) mediated Virus-Induced Gene Silencing (VIGS) (Scofield et al., 2005; Yuan et al., 2011). Silencing was done in the glaucous wheat cultivar Bobwhite, a genotype that has previously been demonstrated to be amenable to BSMV-VIGS (Scofield et al., 2005; Lee et al., 2015b; Lee et al., 2015a). While we could find a fragment that is predicted to silence DMH specifically, silencing of DMP resulted in a possible confounding effect of off-target silencing of sequence-related PKS gene family members. To mitigate the effects of off-target silencing, we used two independent VIGS constructs designed to target DMP for silencing (BSMV:asDMP-1 and BSMV:asDMP-2; see Materials and Methods). Flag leaf sheaths and glumes of newly emerged spikes of BSMV:mcs4D-infected non-silenced control plants were glaucous, while a glossy phenotype was observed in the flag leaf sheaths and glume tissues of wheat plants inoculated with either BSMV:asDMP-1 or BSMV:asDMP-2. A similar glossy phenotype was observed in plants in which TaDMH was targeted for silencing (BSMV:asDMH) (Figure 4 A). Flag leaf sheaths and spikes of BSMV:mcs4D-infected control plants were visually similar to un-infected control plants (Supplemental Figure 5A).

Expression assays of flag leaf sheath samples harvested from emerging spikes [Zadoks GS 45-47 (Zadoks et al., 1974)] confirmed that DMP and DMH transcript levels were significantly reduced in BSMV:asDMP-1-, BSMV:asDMP-2-, and BSMV:asDMH-infected plants, respectively (Figure 4B, C). Scanning electron microscopy revealed significantly fewer epicuticular wax crystals on the flag leaf sheaths and glumes of silenced plants than on the corresponding tissues of control plants (Supplemental Figure 5B). Moreover, β-diketone and hydroxy-β-diketone amounts were significantly reduced in all BSMV:asDMP-1-, BSMV:asDMP-2- and BSMV:asDMH-infected plants (Figure 4D, E), providing further evidence for the role of the DMP and DMH genes in the wheat β-diketone biosynthesis pathway.

A β-diketone metabolic gene cluster is also present on the 2HS barley chromosome arm
The finding of a $\beta$-diketone metabolic gene cluster in the wheat $W1$ locus prompted us to examine the physical location of the $\beta$-diketone biosynthesis genes in barley. The $W1$ region on wheat 2BS is in synteny with the short arm of barley chromosome 2H (Kole et al., 2006). Furthermore, it was previously hypothesised that the barley $\beta$-diketone biosynthesis gene(s) might represent a metabolic gene cluster located on the barley 2HS chromosome [Cer-$c$, Cer-$q$ and Cer-$u$; (von Wettstein-Knowles, 1992)]. Querying the three $\beta$-diketone biosynthesis genes from wheat against the barley genome returned three top hits corresponding to genes MLOC_59804, MLOC_13397 and MLOC_12151 (designated Hv-$DMP$, Hv-$DMH$ and Hv-$DMC$, respectively, see Table 1). These genes are located within an interval of less than 1 Mb on 2HS (the exact cluster size cannot be determined in the current barley genome assembly), indicating a metabolic gene cluster in barley similar to the one in wheat.

The barley cer-$c$, q, and u single mutants possess mutations in either one of the three $\beta$-diketone metabolic cluster genes, which are likely deleted in double and triple mutants

Hundreds of barley cer mutants (Lundqvist and von Wettstein, 1962; Lundqvist et al., 1968; Lundqvist and von Wettstein, 1971) are publicly available, including the cer-$c$, cer-$q$ and cer-$u$ mutants deficient in $\beta$-diketone biosynthesis. To examine if the barley $DMP$, $DMH$, and $DMC$ genes are indeed deficient in the previously characterized cer mutants, coding regions of the three cluster genes were amplified from five cer-$c$, six cer-$q$ and five cer-$u$ single mutants, three double mutants and one triple cer-$cqu$ mutant. Of these, one cer-$c$ mutant (cer-$c$.36) had a premature termination codon (PTC) in the $DMP$ gene, one cer-$u$ mutant (cer-$u$.69) had a PTC in the $DMC$ gene and four cer-$q$ mutants (cer-$q$.42, cer-$q$.56, cer-$q$.82 and cer-$q$.213) had PTCS in the $DMH$ gene. Furthermore, the $DMH$ and $DMC$ genes could not be amplified by PCR from DNA of the double mutant cer-$qu$.813 using different oligonucleotide pairs targeting the three genes. Finally, the $DMP$, $DMH$ and $DMC$ genes could not be amplified in the cer-$cqu$.724 triple mutant with any of the gene-specific oligonucleotide pairs (Figure 5A, Supplemental Figure 6). Expression analysis of the three genes in cer-$cqu$.724 supported a deletion of the entire $\beta$-diketone gene cluster in this triple mutant (Figure 5B-D). In total, eight barley cer mutants deficient in $\beta$-diketone biosynthesis were found to bear mutations or deletions
of the β-diketone biosynthetic genes, firmly supporting that barley DMP, DMH, and DMC are the genes underlying the cer-c, cer-q and cer-u phenotypes, respectively.

The wheat W1 and the barley Cer-cqu gene clusters are conserved

The Zavitan reference genome analysis revealed that the W1/Iw1 region contains many gene duplications (Figure 2C). Beside the more recent duplications, that resulted in highly similar genes (i.e >99.3% identity) and are denoted with a different numerical suffix, there were also probably more ancient duplications, which resulted in less similar genes (i.e, P450-1, P450-2 and P450-3). In order to check whether the whole Zavitan cluster exists in barley, the Zavitan cluster genes were queried against the barley genome (http://www.gramene.org/). Interestingly, we discovered that in addition to the barley homologs of PKS-1, HYD-1, and P450-1 Zavitan genes (MLOC_59804, MLOC_13397, and MLOC_12151, correspondingly), all the other related genes (i.e, PKS-2, PKS-3, HYD-2, P450-2, P450-3) and even the likely unrelated HlyIII gene that resides inside the Zavitan cluster have hits in the barley cluster region (Table 2). This includes all the highly similar Zavitan genes (denoted with a different numerical suffix, see Figure 2C), and had the same hit in barley. Remarkably, the genes that delimit the gene cluster and are not related to β-diketone biosynthesis are also not conserved in the barley cluster. Notably, WES, which is located close to the border or the gene region in Zavitan (see Figure 2C) does not have a hit in the gene cluster region in barley. Interestingly, MLOC_11854 is located within the Cer-cqu region, and it encodes a putative BAHD, a family of acyltransferases previously associated with the formation of cuticular lipids esters (Molina and Kosma, 2015).

E. coli cells expressing barley DMH accumulate predicted intermediates of the β-diketone biosynthesis pathway

Our findings implicated an esterase/hydrolase enzyme in β-diketone biosynthesis, even though such activity had not been predicted to be part of the pathway before (von Wettstein-Knowles, 2012). E. coli cells expressing the barley DMH gene produced two metabolites that were undetectable in the control, C_{15} 2-ketone and C_{15} 2-alkanol. Additionally, C_{14} 3-hydroxyacid was present in low concentration in the control and significantly increased in the lipid extract of cells expressing Hv-DMH (Figure 6 A, for
compound identification see Supplemental Figure 7). C<sub>15</sub> 2-ketone is known to arise from spontaneous decarboxylation of the corresponding C<sub>16</sub> 3-keto fatty acid, either in vivo or during sample preparation and GC-MS analysis (Kornberg et al., 1947). We therefore interpret the ketone as a proxy for the ketoacid and conclude that this acid is the immediate hydrolase product. It seems plausible that both the 3-keto fatty acid and the 2-ketone may have been reduced to 3-hydroxy fatty acid and 2-alkanol by endogenous E. coli enzymes, respectively, thus explaining the other major products identified (Figure 6B). However, it cannot be ruled out that Hv-DMH may have intercepted 3-hydroxy fatty acid elongation intermediates. Since all the major substrate chain lengths occur during plastidial fatty acid synthesis but not during further ER elongation, we suggest that the hydrolase uses 3-ketoacyl-ACP rather than 3-ketoacyl-CoA substrates. DMH may thus be tentatively defined as a medium- and long-chain 3-ketoacyl-ACP thioesterase, however its exact enzyme activity remains to be determined by detailed biochemical characterization.
DISCUSSION

The transition from vegetative to reproductive growth in cereals is a critical developmental phase that determines adaptation to a wide range of environments. This process is accompanied by the accumulation of $\beta$-diketone waxes on surfaces of various glaucous cereal crops. Indeed, glaucousness has been established as an advantage for yield, especially under dry conditions (Fischer and Wood, 1979; Johnson et al., 1983; Febrero et al., 1998; Merah et al., 2000). Glaucousness in wheat is not only governed by the wax production locus $W1$, but is influenced by the dominant epistatic effect of $lw1$ (in hexaploid wheat also $W2$ and $lw2$). The $lw$ genes and their inhibition mechanism are still unknown. The $lw$ genes might be negative regulators, possibly involving RNA silencing similar to the regulation of CER3 in Arabidopsis (Lam et al., 2012; Lam et al., 2015). They could also encode E3 ubiquitin ligases that are specifically involved in $\beta$-diketone protein degradation through the proteasome, or can inhibit $W$ genes through many other mechanisms. We thus anticipate that the elucidation of genes in the wax production locus $W1$ reported here will set the stage for elucidating the wax inhibitor $lw1$ locus.

Multiple genes rather than a single one encoding a multifunctional enzyme are involved in $\beta$-diketone biosynthesis in barley and wheat

The question whether the barley $\beta$-diketone biosynthesis locus is represented by a gene cluster or corresponds to a single multifunctional gene has been a matter of debate in the last three decades. Genetic studies characterizing $\beta$-diketone mutants suggested a closely linked location for the three complementation groups of this pathway ($Cer-c$, $Cer-q$ and $Cer-u$) on 2HS of barley (von Wettstein-Knowles and Søgaard, 1980). In a subsequent report it was shown that the phenotype of double and triple cer mutants could be reverted by additional mutagenesis, suggesting that the $Cer-cqu$ locus most probably corresponds to a multifunctional gene (von Wettstein-Knowles and Søgaard, 1981). Interestingly, among the 434 diketone cer mutants that were generated using seven types of mutagenic treatments, 13 double and triple mutants were found. Eleven out of those 13 double and triple mutants were generated using neutron mutagenesis (von Wettstein-Knowles and Søgaard, 1980). This mutagenesis procedure is known to
cause DNA deletions, ranging in size from kb to Mb (Li et al., 2001; Men et al., 2002), and therefore revertants are highly unlikely to occur. Indeed, here we show a correlation between the barley mutant groups (cer-c, q and u) and mutations in DMP, DMH, and DMC that clearly correspond to a metabolic gene cluster and not to a multifunctional gene. Moreover, deletion mutations in the corresponding genes found in the barley double and triple mutants (i.e. cer-qu.813 and cer-cqu.724) match the finding of a gene cluster. Our results here have been corroborated in a very recent report that described the same three clustered genes to be involved β-diketone biosynthesis in barley (Schneider et al., 2016). Using BAC libraries, Schneider et al. (2016) determined that the barley gene cluster has a size of 101 kb. Furthermore, barley mutants were sequenced and homology-based modelling suggested that many of the mutations affect the protein structure or active-site residues.

**Genes in the wheat W1/lw1 and barley Cer-cqu regions likely encode enzymes involved in the β-diketone biosynthesis and related branching pathways**

Analysis of the W1/lw1 interval in the genome of the wild emmer wheat resulted in the detection of 22 predicted genes (including partial genes and pseudogenes). Sequences of 16 of the 22 genes in the cluster region could be associated not only with the biosynthesis of β-diketones but also with additional wax compounds including methylketones, 2-alkanol esters, and may even be alkylresorcinols (Figure 6). The possible common role of all these compounds is in biotic stress response, acting as bactericides, fungicides or insecticides (Lin et al., 1987; Reiss, 1989; Chatzivasileiadis and Sabelis, 1997; García et al., 1997; Kozubek and Tyman, 1999). The set of 16 genes belong to four different classes including cytochrome P450s, type-III polyketide synthase, hydrolases and the membrane bound O-acyl transferase (MBOAT) family.

The W1/lw1 region is apparently very dynamic, with recent gene duplications as well as more ancient ones, which likely resulted in diverse gene functions. For example, the hydroxylation reactions leading either to 8-/9- or to 25-hydroxy-β-diketone in different wheat and barley genotypes might be catalysed by very similar hydroxylating enzymes with slightly different substrate and/or product specificity. The different CYP450s found in the wheat cluster and associated (by sequence similarity) with two CYP450 subfamilies [i.e. CYP709C1 (DMC/P4501.1, P450-2.1, P450-2.2) and
CYP96A15 (P450-3.1)] are both related to proteins mediating mid-chain hydroxylation and could be catalyzing the synthesis of hydroxy-\(\beta\)-diketones. It is interesting that proteins of both P450 subfamilies (i.e. CYP709C1 and CYP96A15) were found related to several other CYP450 subfamilies whose members are known to be involved with the biosynthesis of plant barriers including cuticular lipids (wax and cutin), suberin and sporopollenin. Among the genes in the \(\beta\)-diketone biosynthesis cluster we also identified a putative \textit{WAX ESTER SYNTHASE (WES)}. As 2-alkanol esters are known to be by-products of the \(\beta\)-diketone pathway (von Wettstein-Knowles, 2012), the involvement of an ester-forming enzyme will be an exciting subject for further investigation. Importantly, both CYP96B30 and WES were found to be highly co-expressed with the three cluster genes, \textit{DMP}, \textit{DMH}, and \textit{DMC}. Recently, Zhang et al. (2015) identified the \textit{W3} locus, which is linked to \textit{W1} and suggested its involvement in \(\beta\)-diketone biosynthesis. Given the complexity of the \textit{W1/lw1} region, \textit{W3} might be included in this interval.

Examining the barley cluster region revealed that in addition to the homologs of wheat \textit{PKS-1}, \textit{HYD-1}, and \textit{P450-1} (barley \textit{MLOC}_59804, \textit{MLOC}_13397 and \textit{MLOC}_12151, respectively) almost all genes in the wheat cluster possess barley homologs in the \textit{Cer-cqu} region. Genes located at the cluster edges that are likely not related to \(\beta\)-diketone biosynthesis (by homology and annotation) are also not conserved in the barley cluster. This further supports our current notion that duplicated genes within the gene cluster are most likely related to \(\beta\)-diketone biosynthesis since there was a strong evolutionary selection pressure to keep the cluster intact in two different plant species. As mentioned above, the current barley genome assembly does not enable to detect the gene order within the cluster and the exact cluster size. Yet, it is clear that the barley homologs for the Zavitan cluster genes are located within the same genomic region. This is supported by the recent paper of Schneider et al. (2016) which found that \textit{MLOC}_13649, \textit{MLOC}_71974 (corresponding to Zavitan P450-2 and P450-3) and \textit{HlyIII} genes are located between two flanking markers of the \textit{Cer-cqu} locus, together with \textit{MLOC}_59804, \textit{MLOC}_13397 and \textit{MLOC}_12151 (corresponding to Zavitan \textit{PKS-1}, \textit{HYD-1} and \textit{P450-1}). \textit{MLOC}_13649 and \textit{MLOC}_71974 were also found to be deleted in several \textit{Cer-cqu} mutants. Further investigation of all clustered genes in
these two plant species will be subject for future research in order to examine their role in the \( \beta \)-diketone pathway and its branches.

**A revised model for the \( \beta \)-diketone biosynthesis pathway**

Based on our characterization of DMH and the likely reactions catalysed by the DMP and DMC enzymes, the \( \beta \)-diketone biosynthesis pathway can now be revisited (Figure 6B). We propose that DMH first intercepts keto-fatty acyl-ACP intermediates from the plastidial fatty acid synthase system. The thioester hydrolysis catalysed by DMH leads to free ketoacids, which are then converted into \( \beta \)-diketone and its hydroxy derivatives by the DMP and DMC enzymes, respectively. Alternatively, the ketoacids may also undergo (either spontaneous or enzymatic) decarboxylation to 2-ketones (methylketones) that serve as precursors for reduction to 2-alkanols and acylation (catalysed by a WES enzyme) to the 2-alkanol esters.

**Activity of DMH as a hydrolase / carboxylesterase and the link to methylketone biosynthesis**

Two alternative hypotheses have been put forward for the activity of the enzyme encoded by barley Cer-\( q \) locus, identified here to be the DMH gene. Originally, it was suggested that the enzyme is localized to the ER, where it functions as a PKS utilizing acyl-CoA substrates to form ketoacyl-CoA intermediates (von Wettstein-Knowles, 2012). The recent discovery that the Cer-\( q \) sequence bears similarity with lipases then prompted speculation that the enzyme instead hydrolyses hypothetical glycerolipid intermediates into free acids (Schneider et al., 2016).

However, our experiments testing the DMH activity by heterologous expression in *E. coli* led to accumulation of C\(_{15}\) 2-ketone (C\(_{15}\) methylketone) which is most probably a decarboxylation product of C\(_{16}\) 3-ketoacid. The product of the DMH–catalysed reaction is thus a free ketoacid, rather than the previously assumed acid lacking the keto group. Conversely, this finding strongly suggests that the substrate of the reaction is a ketoacyl compound present in plants as well as *E. coli*. Finally, based on the sequence similarity with hydrolases and carboxylesterases, we conclude that DMH most likely functions as a thioesterase intercepting common ketoacyl intermediates of fatty acid synthesis. Since the acyl chain lengths involved are known to be generated in
plants by the plastidial fatty acid synthase (FAS) system, it seems plausible that the ketoacyl-ACP is intercepted between the ketoacyl-ACP synthase (KAS) and ketoacyl-ACP reductase (KAR) steps. An alternative interception of a ketoacyl-CoA intermediate of the ER-localized fatty acid elongase (FAE) system seems unlikely, since they are thought to elongate only acyl chains beyond C\textsubscript{16}, longer than the chain lengths observed in the \textit{E. coli} experiment.

Methylketones are known to be side products of the \textit{β}-diketone biosynthesis pathway (von Wettstein-Knowles, 2012). Previous studies demonstrated that methylketone biosynthesis in \textit{Solanum habrochaites} (\textit{Sh}) involves two enzymes, methylketone synthase 1 and 2 (MKS1 and MKS2) (Fridman et al., 2005; Ben-Israel et al., 2009; Yu et al., 2010; Auldridge et al., 2012). While MKS2 (a HotDog fold protein) acts as a thioesterase hydrolysing (primarily C\textsubscript{12} and C\textsubscript{14}) \textit{β}-ketoacyl-ACPs to generate the corresponding 3-ketoacids, MKS1 (a α/β-hydrolase fold protein) is a decarboxylase that converts the 3-ketoacids to the corresponding (C\textsubscript{11} and C\textsubscript{13}) 2-ketones (Yu et al., 2010). The tomato study further revealed that decarboxylation occurs either enzymatically (catalysed by MKS1) or spontaneously, however with slow rate (Yu et al., 2010). Taken together, the tomato results thus underpin our conclusion that DMHs (in wheat and in barley) act as thioesterases that hydrolyse \textit{β}-ketoacyl-ACPs to 3-ketoacids, which are then spontaneously/ enzymatically decarboxylated to 2-ketones.

Our wheat wax analyses confirmed earlier results for barley waxes, showing that the amounts of \textit{β}-diketones are much higher than those of the accompanying 2-alkanol esters, the final products derived from methylketone intermediates. These findings suggest that the overall pathway flux is higher towards \textit{β}-diketone products then to the 2-ketone intermediates en route to the 2-alkanol ester side products. Therefore, relatively slow, spontaneous decarboxylation of the common ketoacid intermediate may be sufficient to account for the formation of low amounts of 2-alkanol esters. Yet, alternative scenarios for the decarboxylation reaction cannot be excluded at present. On the one hand, a separate, unknown decarboxylase may exist, which might even be encoded within the gene cluster. On the other hand, DMH might act as a bifunctional enzyme having both hydrolase and decarboxylase activities, similar to other enzymes.
like Polyneuridine Aldehyde Esterase (PNAE) from *Rauvolfia serpentina*; (Dogru et al., 2000; Yang et al., 2009)].

Finally, it should also be noted that both tomato proteins involved in formation of 2-ketones (MKS1 and MKS2) localized to the plastid, even though MKS1 lacks a typical transit peptide (Fridman et al., 2005; Yu et al., 2010) as is the case with the wheat and barley DMH proteins. It is worth mentioning that, despite the lack of typical transit peptides, the plant-mPLoc tool (Chou, 2005; Shen and Chou, 2006; Chou and Shen, 2007, 2008, 2010) predicts that the wheat and barley DMHs localize to the chloroplast or the nucleus, but not the ER. Surely, further studies are required in order to fully characterize the DMH enzyme activity and its sub-cellular localization in both barley and wheat.

**Common and unique features of the β-diketone metabolic gene cluster**

The β-diketone biosynthesis clusters found here in the wheat *W1* and barley *Cer-cqu* loci join a growing number of plant metabolic gene clusters discovered in the past years (Osborn, 2010; Boycheva et al., 2014; Nützmann and Osbourn, 2014). These clusters clearly share a few typical features detected in other metabolic gene clusters to date. These include: (i) a typical size of a several hundred kb (800 kb in wheat), (ii) the presence of at least three genes, (iii) the involvement of genes encoding a minimum of three types of biosynthetic enzymes, and (iv) the tightly co-expression of the clustered genes (Nützmann and Osbourn, 2014; Nützmann et al., 2016). Another common feature of plant metabolic gene clusters is the presence of an enzyme catalysing the first committed step in the pathway. Such enzymes divert the pool of an intermediate metabolite from core/primary pathways into secondary/specialized metabolism. In the case of terpenoid-producing clusters, triterpene and diterpene cyclases draw substrates from the core cytosolic and plastidic isoprenoid pathways, respectively (Qi et al., 2004; Wilderman et al., 2004; Field and Osbourn, 2008; Sawai and Saito, 2011). Other examples include the first committed enzymes in the biosynthesis of Dhurrin, a cyanogenic glycoside from *Sorghum bicolor* and the cyclic hydroxamic acid 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) from maize, both utilizing substrates from core amino acid biosynthetic pathways (Frey et al., 1997; Takos et al., 2011). In the case of the β-diketone biosynthesis cluster, DMH likely performs the first
committed step intercepting ketoacyl-ACP intermediates from the core fatty acid synthase (FAS) system. In terms of enzyme activities, the β-diketone cluster includes for the first time members of the hydrolase/ carboxylesterase and PKSs families, the latter often found in bacterial metabolic clusters (Shen, 2003; Wang et al., 2014a).

Boutanaev et al. (2015) proposed that metabolic gene clusters are often located in unstable chromosomal regions (close to the telomere or in highly dynamic regions, i.e. rich in transposable elements). The clustering of genes in highly unstable regions probably reflects the advantage conferred by coinheritance of beneficial gene combinations. Both $W1$ in wheat and $Cer-cqu$ in barley are located in sub-telomeric regions, and $W1$ is indeed a very dynamic region. Therefore, it seems that through evolution glaucousness was favored and inheritance of the genes as a cluster was established, at least in wheat and barley.

While common in bacterial and fungal clusters of natural product biosynthesis, to date none of the plant metabolic gene clusters appear to contain regulatory genes controlling the corresponding pathway. In a recent example, Cárdenas et al. (2016) demonstrated that an AP2-type transcription factor (GLYCOALKALOID METABOLISM 9; GAME9) could likely activate genes of the clustered steroidal glycoalkaloid (SGA) pathway genes such as GAME4. Yet, GAME9 is located on chromosome 1 and hence not physically associated with the SGA cluster genes situated on chromosomes 7 and 12 in tomato and potato. The wheat β-diketone cluster could turn out to be the first to contain a regulatory gene, as the $Iw1$ dominant wax inhibitor resides in the vicinity of the $W1$ locus and it might encode a negative regulator of the β-diketone pathway.

In this study, we used diverse approaches to shed light on the molecular basis of β-diketone production in wheat and barley. The importance of cuticular lipids including β-diketones to cereal crop performance has been demonstrated several times by now including the control of the covered/ hulled and naked caryopsis phenotype as well as leaf water conservation (Taketa et al., 2008; Chen et al., 2011). Hence, the findings here extend the current toolbox allowing breeding and manipulation of surface traits in major cereal crops for improved biotic and abiotic stress response and ultimately yield.
MATERIAL AND METHODS

Plant material

BL and CASL*2Bs lines were generously provided by Moshe Feldman, Weizmann Institute of Science. Barley (Hordeum vulgare) accessions were kindly provided by the NordGen gene bank (http://www.nordgen.org/index.php/en). Plants used for scanning electron microscopy imaging, wax analysis, transcriptome libraries and mutant screen were grown in greenhouse (22°C during day time and 20°C during the night). Two F2 populations were used in the current study: (i) tetraploid population derived from a cross between the glossy wild emmer wheat (Triticum turgidum subsp. Dicoccoides) accession 'TTD140' collected from Rosh-Pina, Israel, and glaucous durum wheat Triticum durum cv. Svevo. (ii) BL (Triticum aestivum ssp. aestivum cv. Bethlehem [BL]) crossed with CASL*2BS. The F2 populations were grown during the winter season (November 2013 - May 2014) at the experimental farm of the Hebrew University in Rehovot, Israel. For the VIGS experiments, Nicotiana benthamiana plants for preparation of BSMV sap inoculum and wheat (Triticum aestivum) cultivar Bobwhite were raised in Levington F2+S Seed & Modular Compost Plus Sand and Rothamsted prescription mix compost (Petersfield Products, UK), respectively, in a controlled environment room with day/night temperature of 23°C/ 20°C, 60% relative humidity, and a 16 hours photoperiod (approximately 180 μmol.m^-2.s^-1 light intensity).

Scanning electron microscopy (SEM) imaging

Leaf sheaths and peduncles of plants in Zadoks ~GS59 were sampled for cryo-SEM. Samples were freshly cut on narrow strips, placed in specially designed cryo-holder and quickly frozen in liquid nitrogen. The frozen samples were kept in liquid nitrogen and transferred by using a vacuum cryotransfer device (VCT 100; Leica Microsystems) to a freeze-fracture device (BAF 60; Leica Microsystems). Samples were etched for 20 min at ~95°C, and coated with 6 nm platinum/carbon by double-axis rotary shadowing. Samples were observed at ~120°C in an Ultra 55 SEM (Zeiss) by using a secondary electron in-lens detector. Cryo-scanning electron microscopy images of glume and flag leaf sheath surfaces of plants infected with VIGS constructs were taken using a JEOL JSM-6360 LVSEM for 500x magnification images. Small (~5 mm) sections of the flag
leaf sheath or glume tissue were cut using a scalpel and mounted on an aluminium stub using a 50:50 mix of Tissue-Tek tissue freezing medium and colloidal graphite. The samples were rapidly frozen in liquid nitrogen then transferred to the GATAN Alto 2500 cryo-preparation chamber. They were etched at -95°C for 2 min to remove any ice contamination then coated with a layer of gold/palladium (Au/Pd) once the temperature had recovered to -150°C. Samples were then transferred to the SEM which was maintained at -150°C and imaged using an accelerating voltage of 5 kV.

**Preparation of wax extracts**

Flag leaf blades or leaf sheath together with peduncles were collected from BL and CASL*2BS lines, and surface areas were measured for normalization between samples. Each experiment had ≥ four biological replicates. Samples were submerged in a 20 ml scintillation vial containing 10 ml CHCl₃ (Aldrich, ≥99%, 0.75% ethanol as stabilizer) and 5 μg of n-tetracosane (Alfa Aesar, ≥99%) as internal standard. Samples were gently shaken at room temperature for 30 sec, and then the chloroform was transferred to another vial. A fresh portion of 10 ml CHCl₃ was added, the solution was shaken for another 30 sec, and then the two extracts were combined and concentrated under a gentle stream of N₂ (Praxair, ≥99.998%), before finally being transferred to 2 ml GC sampler vials. VIGS plant wax extraction was done as above, with few modifications: Leaf sheaths were collected, surface areas were measured and 4 ml of CHCl₃ was used twice for wax extraction. Samples were kept open in a chemical hood for CHCl₃ evaporation until dryness. Each sample was re-dissolved in 1 ml CHCl₃ containing 0.5 µg of n-tetracosane as internal standard, vortexed for 15 sec, concentrated to 100 µl under a gentle stream of N₂ and transferred to 2 ml GC sampler vials.

**Qualitative and quantitative analyses of wax extracts**

BL and CASL*2BS wax samples were derivatized by refluxing in 10 µl N,O-bis(trimethylsilyl) trifluoroacetamide (Aldrich, GC grade) and 10 µl pyridine (Aldrich, ≥99.8%, anhydrous) at 70°C for 30 min, and then removing excess reagents under nitrogen. Once dry, samples were re-dissolved in 50 µl of CHCl₃ for GC analysis. Two GC instruments were used for separation and detection of wax constituents, both equipped with the same type of capillary GC column (6890N, Agilent; HP-1 column; 30
m length, 0.32 mm inner diameter, 0.1 µm film thickness) using on-column injection and following the same temperature program (2 min at 50°C, ramp 40°C min⁻¹ to 200°C, constant for 2 min, ramp 3°C min⁻¹ to 320°C, constant for 30 min). The first employed Helium gas (Praxair, ≥99%) as mobile phase, at a flow rate of 1.4 ml min⁻¹, and was equipped with MS detector (5973N, Agilent, EI-70 eV), serving primarily the purpose of qualitative identification of separated wax compounds. The second used H₂ carrier gas (Praxair, ≥99.95%) at 2.0 ml/min and an FID detector, for quantification of individual wax homologs based on normalization of peak areas against that of the internal standard. The relative response factors of all cuticular wax classes with respect to the internal standard were approximated to 1.00, in agreement with Riederer and Schneider (1989).

VIGS and E. coli samples (concentrated to 100 µl CHCl₃) were derivatized by adding 20 µl N,O-bis(trimethylsilyl)trifluoroacetamide and 20 µl pyridine at 70°C for 40 min. Sample volume of 1 µl was injected in splitless mode on a GC-MS system (Agilent 7683 autosampler, 7890A gas chromatograph and 5975C mass spectrometer). GC was performed (Zb-1ms column; 30 m length, 0.25 mm inner diameter, 0.50 µm film thickness; Zebron, Phenomenex) with injection temperature 270°C, interface set to 250°C and the ion source to 200°C. Helium was used a carrier gas at constant flow rate of 1.2 ml min⁻¹. The temperature program was 0.5 min isothermal at 70°C, followed by a 30°C min⁻¹ oven temperature ramp to 210°C and a 5°C min⁻¹ ramp to 330°C, then kept constant during 21 min. Mass spectra were recorded with an m/z 40–850 scanning range. The chromatograms and mass spectra were evaluated using the MSD Chemstation software (Agilent). E. coli samples were injected as the VIGS samples with the following adaptations: Sample volumes of 1 µl were injected with a split ratio of 50:1. Injection temperature was 250°C, the interface set to 250°C and the ion source to 200°C. The temperature program was 0.5 min isothermal at 120°C, followed by a 30°C min⁻¹ oven temperature ramp to 200°C, then ramped by 5°C min⁻¹ to 250°C and kept for 21 min. For quantification of β-diketones and hydroxy-β-diketones, the GC peak areas of both the derivatized enol tautomers and the non-derivatized beta-diketo forms were added together, as these compounds are not derivatized uniformly. Hydroxy-β-diketones included the 8- and 9-hydroxy isomers.
Expression in *E. coli* cells and lipid extraction

Barley *DMH* (MLOC_13397) was cloned into the pET28-TEVH plasmid (Peleg and Unger, 2008) using restriction-free cloning procedure (Unger et al., 2010) to generate the pET28-Hydrolase plasmid. Oligonucleotides are listed in Supplemental Table 4. pET28- Hydrolase and pET28- TEVH (control) were transformed into BL21 *E.coli* cells. Five colonies of each transformed *E. coli* were grown in 5 ml LB supplemented with kanamycin in 37°C overnight. Each sample was diluted 1:100 and grown to OD600 ~0.6-0.8 in 10 ml LB+ kanamycin. Cells were induced with 500 μM IPTG at 15°C overnight. The cells were washed with phosphate buffered saline (PBS) and kept at -80°C until further use. *E. coli* cell pellets were re-suspended in 1ml of double-distilled water (DDW), transferred to glass vials and 3.75 ml of chloroform: methanol (1:2) mixture supplemented with 1 μg of *n*-tetracosane internal standard were added to each sample. Samples were vortexed for two hours and sonicated for 10 min. To each sample 1.25 ml of chloroform was added, the sample vortexed and 1.25 ml of DDW were added followed by a second sonication for 10 min. The samples were centrifuged for 5 min at 3000 rpm, and lower phase recovered. To extract the remaining lipids, 1.9 ml of chloroform was added to the non-lipid upper phase, following vortex and centrifugation for 5 min at 3000 rpm. The two organic phases were combined in the glass tube and concentrated to 100 μl under a gentle stream of N2. Samples were stored at −20°C until derivatization.

Transcriptome sequencing, assembly and analysis and Reverse Transcription Quantitative PCR (RT-qPCR)

Wheat BL and CASL*2BS* libraries for Illumina (Illumina Inc., San Diego, CA, USA) high-throughput strand-specific RNA sequencing (RNA-seq) were prepared as follows: total RNA was extracted with the TRIzol method using standard TRIzol RNA extraction protocol. For each sample, 5 mg of RNA were used for preparation of RNA-seq libraries as described in Zhong et al. (2011) with minor modifications. Briefly, poly (A) RNA was isolated from total RNA using Dynabeads Oligo (dT)$_{25}$ (Invitrogen), fragmented at 94°C for 5 min and eluted. Single strand cDNA was synthesized using random primers, dNTPS and SuperScript III (Invitrogen) reverse transcriptase. Second strand cDNA was
generated using DNA polymerase I (Enzymatics Inc., Beverly, MA, USA) and dATP, dCTP, dGTP and dUTPs. After end-repair (Enzymatics), dA-tailing with Klenow 3′-5′ (Enzymatics) and adapter ligation (Quick T4 DNA Ligase; New England Biolabs, Ipswich, MA, USA), the second strand (dUTP-containing) was digested by uracil DNA glycosylase (Enzymatics). The remaining first strand adaptor-ligated cDNA was used for 13–15 cycles of PCR enrichment with NEB-Next High-Fidelity PCR Master Mix (New England Biolabs). Sequencing of 12 wheat libraries (BL, CASL*2BS and F2 progeny of the cross BL X CASL*2BS) was performed on two lanes of HiSeq 2500 using v3 clustering and sequencing reagents; six libraries were multiplexed on each lane (two libraries are used in this study). The sequencing was run using RTA 1.17.20 and analyzed by CASAVA 1.8.2, resulting in 14-16 million passed filter 100 bases paired end reads per library. Sickle (Joshi and Fass, 2011) was used (in its paired end mode) for trimming the reads. Sequences shorter than 50 bases after trimming were discarded (only 3′ trimming was allowed). The RNA-Seq reads were combined and were assembled into transcripts using Trinity (version trinityrnaseq-r2013-02-25) (Grabherr et al., 2011) with the option -SS_lib_type RF. A total of 396,155 transcripts and 157,340 components were obtained. RSEM (Li and Dewey, 2011) was used for abundance estimation of the components. The obtained transcripts were annotated using Blast2GO (Conesa et al., 2005). The counts were TMM-normalized using edgeR (Robinson and Oshlack, 2010) and TMM-normalized FPKM matrix was calculated using Trinity’s script run_TMM_normalization_write_FPKM_matrix.pl. Two libraries were used in this study- BL and CASL*2BS from peduncle tissue. Hits for the RNAseq transcripts of barley and wheat genes were received from blast in http://www.gramene.org/. For RT-qPCR, RNA was extracted with the TRIzol method using standard TRIzol RNA extraction protocol. DNase treatment and reverse-transcription reactions were performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions. Primers are listed in Supplemental Table 4. Fast SYBR Green reagent and StepOnePlus instrument (Applied Biosystems) were used for RT-qPCR. Reaction steps: initial step in the thermal cycler for 20 sec at 95°C, followed by PCR amplification for 40 cycles of 3 sec at 95°C and 30 sec at 59°C, and finally dissociation analysis to confirm the specificity of PCR products. Total reaction
volume was 10 µl, containing 2.5 µM of each primer. At least three biological replicate
were analysed for each biological group. Relative transcript levels were calculated
according to the ΔΔCt method (Livak and Schmittgen, 2001). Normalization was done
by the following reference genes: Wheat Actin for RT-qPCR in BL and CASL*2BS
internodes, barley Actin for Bonus and cer-cqu.724 and CDC48 for VIGS experiment
(see Supplemental Table 4).

**CYP450 nomenclature**

The wheat CYP450 proteins in this study were kindly annotated by Dr. David R. Nelson,
University of Tennessee, Memphis, TN, USA as follows: wheat DMC (cv. BL)-
CYP709J4, Zavitan P450-1.1- CYP709J4 ortholog, Zavitan P450-2.1- CYP709J5
(partial), Zavitan P450-3.1- CYP96B30.

**Gene co-expression analysis**

Wheat transcriptome data (https://urgi.versailles.inra.fr/files/RNASeqWheat/) of five
organs during three developmental stages was used for co-expression analysis of the
cluster genes. Reads were aligned to the reference transcriptome (created by trinity,
see ‘Transcriptome sequencing, assembly and analyses’ in Materials and Methods).
Expression levels were estimated using RSEM (Li and Dewey, 2011). Cluster genes
were used as “baits” for the co-expression analysis. The analysis was preformed using
the Pearson correlation coefficient with r-value ≥ 0.85.

**Development of molecular markers on the W1 and the Iw1 region**

PCR markers were developed from selected 90K SNPs showing polymorphism
between the parental lines using TaqMan SNP assay (De la Vega et al., 2005) or high
resolution melt (HRM) methods (Vossen et al., 2009). In addition, three
presence/absence markers (wheat DMC, DMP, and DMH) were developed based on
results from the RNAseq experiment described above. Sequence data were obtained
from the International Wheat Genome Sequencing Consortium (IWGSC) website and
from the wild emmer wheat genome assembly (unpublished data) of the *T. dicoccoides*
accession 'Zavitan'. Specific primers were designed using 'Primer3'
(http://bioinfo.ut.ee/primer3-0.4.0/). Sequence alignments were performed using NCBI-
BLAST in order to match the 90K markers to their corresponding genes. BLAST hits were filtered to e-value threshold of 0.0001, >99% identity (sequence similarity/match) and more than 75% of length. Two closely linked EST markers (CD893659 (Xu et al., 2015), JIC009 (Adamski et al., 2013) and three SSR markers: Xgwm614 (Röder et al., 1998), Xwmc661 and Xwmc764 (Somers et al., 2004) were also integrated into the current mapping effort. Sequences of all the markers are available in Supplemental Table 2.

**Annotation of the W1 interval and Genotyping with the 90K iSelect SNP Chip**

Genomic sequence of chromosome 2B of wild emmer accession Zavitan was annotated between the flanking genetic markers of W1, JIC007 and JIC010. As a first step the transposable elements (TEs) were annotated by hand using the TREP database and the non-redundant nucleotide collection hosted on NCBI. The remaining sequence was then searched for open reading frames (ORFs) using the Fgenesh algorithm. The putative genes were translated and compared to the non-redundant protein database of NCBI. In general, ORFs were considered as real genes if their translated protein contained at least one complete protein domain. In addition, the predicted ORFs were tested against the IPK barley BLAST databases “assembly_WGSMorex”, “HC_genres_CDS_Seq” and “full length cDNA” using BLAST (http://apex.ipk-gatersleben.de/apex/?p=284:10:32887702692733::NO). The presence of an orthologous locus in barley was not a requirement to call a predicted ORF a real gene.

Genotyping procedure for the 90K Chip was performed on the parental line DNAs (BL, CASL*2BS, Svevo and TTD140) as described by Wang et al. (2014b) at the UC Davis Genome Center. Genotypic clusters for each SNP were determined using the manual option of GenomeStudio version 1.9.4 (Illumina), based on the data from all of the described genotypes. For gene sequences see ‘Accession Numbers’, below.

**Generation of phylogenetic trees**

Protein sequences were aligned using MUSCLE web tool (http://www.ebi.ac.uk/Tools/msa/muscle/) with default parameters and Fasta output format. Maximum likelihood phylogenetic tree was created using MEGA6 (for P450 tree
in Figure 2D) (Tamura et al., 2013) or MEGA7 (for Supplemental Figure 4) (Kumar et al., 2016), both with the Jones-Taylor-Thornton matrix based substitution model and a 100 bootstrap replications. P450s proteins in the tree (Figure 2D) included TaDMC, six Zavitan P450s, Barley DMC homolog (MLOC_12151) and P450s with known activity (Kandel et al., 2005; Greer et al., 2007; Pinot and Beisson, 2011). Tree was rooted using CYP79A1 from *Sorghum bicolor* as an outgroup. Text files corresponding to the alignments used can be found in Supplemental Data Sets 2-4.

**Construction of three BAC libraries from TTD140**

High molecular weight (HMW) DNA was prepared from 20 g of young leaves of *Triticum turgidum* accession “TTD140” as described by Peterson et al. (2000) Embedded HMW DNA was partially digested with *Hind*III (New England Biolabs, Ipswich, Massachusetts), subjected to two size selection steps and ligated into pIndigoBAC-5 *Hind*III-Cloning Ready vector (Epicentre Biotechnologies, Madison, Wisconsin). Pulsed-field migration programs, electrophoresis buffer, and ligation desalting conditions were performed according to Chalhoub et al. (2004). The first resulting BAC library was named Ttu-B-TTD140. It consisted of 332,160 BAC clones with mean insert size of 126 kb, which represented ~3.3-fold coverage of total TTD140 genome. This first BAC library was classically arrayed into 865 384-well microtiter plates. High-density colony filters were prepared for this whole genomic BAC library. Labeling and hybridization (with 33P) were performed as described in Gonthier et al. (2010). Positive BAC clones were validated individually by PCR amplification using the primer pairs used for probes synthesis. Two additional BAC libraries named respectively Ttu-B-TTD140-ng (*Hind*III restriction) and Ttu-B-TD140e-ng (*Eco*RI restriction) were constructed from the same plant material using a non-gridded BAC library protocol based on Isidore et al. (2005) with the following modifications: growth of pooled BAC clones in liquid LB medium; BAC pools DNA amplification by Whole Genome Genomiphi.v2 phi29 enzyme kit (GE Healthcare) instead of DNA extraction; use of secondary pooling steps in order to identify positive clone coordinates with less screening effort after clone picking. HMW DNA extractions and sizing steps were processed as for the first classical BAC library. For Ttu-B-TTD140-ng 461,540 BAC clones were produced (117 kb average insert size).
representing ~4.5-fold coverage of genome. For Ttu-B-TTD140-ng 652,427 BAC clones
(103 kb average insert size) were produced representing ~5.6-fold coverage of genome.
BAC clones were divided in a total of 1,328 pools before overnight growth and DNA
amplification. Diluted pools were screened using specific PCR markers previously
developed. All these BAC library resources and dedicated screening tools are available
upon request (http://cnrgv.toulouse.inra.fr).

**BSMV-VIGS of β-diketone biosynthesis genes**

The BSMV-VIGS system described by Yuan et al. (2011) comprising three T-DNA
binary plasmids, pCaBS-α, pCaBS-β, and pCa-ybLIC, was used in this study. In silico
predictions by the si-Fi (siRNA Finder, http://labtools.ipk-gatersleben.de) software were
used to select the most effective gene-specific fragments for silencing, ranging from 362
to 387 bp in size. According to si-Fi results it was not possible to identify a region of the
wheat *DMP* gene that would silence *DMP* alone. In order to mitigate the possible
confounding effects of off-target silencing of other PKS family members, two non-
overlapping fragments of the *DMP* gene were cloned separately to generate two
independent VIGS constructs designed to target *DMP* for silencing (VIGS constructs
BSMV:asDMP-1 and BSMV:asDMP-2). DMP-1, DMP-2 and DMH fragments were first
amplified from BL cDNA (For primers see in Supplemental Table 4) and cloned into
pGEM-T (Promega) vector, following by cloning into pCa-ybLIC in antisense orientation
using a ligation-independent cloning strategy. BSMV:mcs4D, which contains non-coding
sequence was included in all VIGS experiments as a non-silencing virus control.
Transformation of the BSMV binary plasmids into *Agrobacterium tumefaciens*
strain GV3101 and agroinfiltration into 3-4 week-old *N. benthamiana* plants was carried out as
described previously (Lee et al., 2014). Sap from the infiltrated *N. benthamiana* leaves
was used to mechanically inoculate the third fully-expanded leaf of 28-day-old wheat
plants at the four-leaf stage. Samples for RT-qPCR analysis to determine target gene
silencing success were harvested from the flag leaf sheaths of spikes in early boot
stage (GS45-47). A minimum of three independent samples per virus treatment, each
sample harvested from an individual plant, were analysed. Flag leaf sheath tissue
samples for GC-MS analysis of wax extracts were taken from fully emerged wheat
spikes prior to anthesis (corresponding to GS58-59). A minimum of four independent
samples per virus treatment were analysed for each tissue type, each sample harvested
from an individual plant.

**Barley cer mutant screen**

Barley $\beta$-diketone cer mutants were ordered from the NordGen bank. The cDNAs
corresponding to the cluster genes; *DMP*, *DMH*, and *DMC* were amplified in the 20 cer
mutants (Figure 5A) and two corresponding wild-type cultivars Bonus and Foma.
Oligonucleotides used for amplification are listed in Supplemental Table 4. The cDNAs
were amplified using OptiTaq DNA Polymerase (EURX), sequenced and compared to
the relevant WT cultivar.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or
GenBank/EMBL databases under the following accession numbers: <To be added in
proof,>.

**SUPPLEMENTAL DATA**

**Supplemental Figure 1.** General scheme of biosynthetic pathways generating different
cuticular wax components.

**Supplemental Figure 2.** Illustration of wheat parental and chromosome arm
substitution lines used in this study.

**Supplemental Figure 3.** Amplification of markers developed for the three candidate
genes in glaucous and non-glaucous wheat.

**Supplemental Figure 4.** Phylogenetic trees of Zavitan proteins.

**Supplemental Figure 5.** Virus-induced gene silencing (VIGS) of the $\beta$-diketone genes
reduces wax accumulation and deposition of epicuticular wax crystals.

**Supplemental Figure 6.** The barley *cer-qu.813* double and *cer.cqu-724* triple mutants
have deletions that include the metabolic gene cluster genes.

**Supplemental Figure 7.** Compound identification of *E-coli* expressing *HvDMH*
metabolites.
Supplemental Text 1. Fine genetic map of 2BS W1/Iw1 loci in different segregating populations.


Supplemental Text 3. Comparison of the W1/Iw1 interval between the wild emmer accessions TTD140 and Zavitan.

Supplemental Table 1. Linked deleted markers in the TTD140 accession detected by the 90K chip analysis.

Supplemental Table 2. Markers and oligonucleotides used for linkage analysis.

Supplemental Table 3. Homology between Zavitan, TTD140 and the corresponding barley genes.

Supplemental Table 4. Oligonucleotides used in this study.

Supplemental Data Set 1. Co-expressed genes using the three β-diketone genes as 'baits'.

Supplemental Data Set 2. Text file of alignments corresponding to the phylogenetic tree of PKS proteins in Supplemental Figure 4.

Supplemental Data Set 3. Text file of alignments corresponding to the phylogenetic tree of hydrolase proteins in Supplemental Figure 4.

Supplemental Data Set 4. Text file of alignments corresponding to the phylogenetic tree of CYP450 proteins in Supplemental Figure 4.

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**AUTHOR CONTRIBUTION**

SHA designed and performed the research and wrote the article. OS performed molecular characterization and fine mapping. RR performed BL and CASL*2BS wax analysis, assisted in GC-MS VIGS experiment analysis and wrote the article. WSL performed VIGS assays, assisted in VIGS analysis and wrote the article. N-Adamski analyzed the physical maps, performed fine mapping and wrote the article. SM assisted with GC-MS operation and analysis. EA assisted in the RNA-seq analysis and performed co-expression. ML performed co-expression. SV and HB performed BAC screening and sequencing. GF analyzed the RNAseq data. EK conducted the BL and CASL*2BS electron microscopy experiments. GBZ was involved with generation of the wild emmer wheat reference genome. N-Alkan assisted in the experiments. CU and KK designed part of the research and wrote the article. AA, AD and RJ designed the research and wrote the article.

**Competing financial interests**

The authors declare no competing financial interests.
FIGURE LEGENDS

Figure 1. Differences in wax phenotypes between the glaucous BL and the glossy CASL*2BS wheat lines. (A) BL is covered with bluish-grey, waxy material while CASL*2BS is glossy. (B) The epicuticular wax covering peduncle and flag leaf (abaxial side) tissues was examined using Cryo Scanning Electron Microscopy. BL and CASL*2BS lines exhibit different wax amounts and crystals shapes. Tubule-shaped crystals predominate on BL while crystals on CASL*2BS are mostly platelet-shaped. Magnification: 10 k. Scale bar indicates 2 µm. Chemical compositions of wheat BL and CASL*2BS wax mixtures on (C) flag leaf blade and (D) flag leaf sheath plus peduncle were determined by GC-MS analysis. Values indicate the mean ±SD of n≥ 4 biological replicates. * p < 0.05, ** p < 0.01, *** p < 0.005.

Figure 2. Genetic and physical maps of W1/Iw1. (A) Location of the locus responsible for the dominant glaucous phenotype (W1) based on analysis of 9,550 gametes from the AUS2499 x Kofa+Lr19 cross. W1 is contained in a 0.16 cM interval on wheat chromosome 2BS flanked by markers JIC009 and JIC010 (B). The locus responsible for the dominant glossy phenotype (Iw1) on wheat chromosome 2BS is found in a similar location based on analysis of 598 gametes from the Svevo x TTD140 cross. Iw1 is completely linked to JIC009 and to the three candidate genes Wheat DMP, DMH, and DMC. Underlined markers represent dominant markers present in Svevo and absent in TTD140. (C) Wild emmer Zavitan (glaucous) and TTD140 (glossy) physical maps. Gene content of Zavitan across the W1 genetic interval is shown. Out of 22 genes, 15 correspond to either cytochrome P450s (P450; pink), type-III polyketide synthases (PKS; blue) or hydrolase (HYD; green) genes (including partial genes and pseudogenes). Genes are ordered based on the Zavitan genome and are numbered accordingly on the Zavitan physical map (middle). Duplicated genes with >99% sequence identity are denoted with the same color and with a different numerical suffix. Pseudogenes are denoted with a psi symbol (ψ). Partial genes are denoted with triangle symbol (▲). The Zavitan best homologs for DMP (two duplicated genes DMP-1.1 and DMP-1.2), DMH (two duplicated genes DMH-1.1 and DMH-1.3) and DMC are shown.
Among the 22 Zavitan genes, WES and $P450\text{-}3.1$ were annotated as wax ester synthase from the MBOAT family (in turquoise) and CYP96B30 (in dark pink), correspondingly, suggesting a possible involvement in wax biosynthesis. The TTD140 physical map (top) shows a common haplotype with Zavitan in the regions surrounding flanking markers JIC036 and JIC032 (dashed black line). However, apart from the multiple local duplications of the NB-Arc-like genes (gene 1 and 2, duplications are indicated in black asterisks), all other sequences and genes from DUF4220 to Ank_PGG are unique to the Zavitan $W1$ haplotype (orange line) compared to the TTD140 $Iw1$ haplotype (light green line). Black vertical lines in the TTD140 physical map are genes exclusive to TTD140. Numbers in purple indicate the number of observed recombinations. A detailed description of the region is provided in Supplementary Text 2, Supplementary Text 3. (D) Phylogenetic tree of wheat DMC (purple) identified from BL and its homologs from Zavitan accession and from barley, with additional P450s from related clades. TaCYP709C1 and AtCYP96A15 (MAH1) are known to catalyze in-chain hydroxylation and are related by sequence to Ta-DMC, Zavitan P450(1-3), barley MLOC_12151 and MLOC_71974. Proteins related to TaCYP709C1 and AtCYP96A15 are shown in pink and blue, respectively. Species abbreviation: At – *Arabidopsis thaliana*, Ph – *Petunia* hybrid, Sb - *Sorghum bicolor*, St – *Solanum tuberosum*, Vs – *Vicia sativa*, Nt – *Nicotiana tabacum*, Ta - *Triticum aestivum*, Hv - *Hordeum vulgare*, Ht – *Helianthus tuberosus*, Os – *Oryza sativa*, El – *Euphorbia lagaesae*, Zm – *Zea mays*. Known enzymatic activities are shown: $\omega h$ – $\omega$-hydroxylation, ich – in-chain hydroxylation, df – diacid formation, ep – epoxidation. Physiological role associated with fatty acid biosynthesis is shown: Cutin – cutin synthesis, Estolide – estolide synthesis, Suberin – suberin synthesis, Sporopollenin – sporopollenin synthesis, Seed oil – seed oil synthesis. Wax- wax synthesis, FA- fatty acid hydroxylation. Genes discussed in this study are underlined.

**Figure 3.** The three $\beta$-diketone metabolic gene cluster members are highly expressed in glaucous tissues and have very similar expression patterns. (A) A wheat tiller in the stem elongation stage in which the upper internode is already glaucous while the next lower internode is still glossy (Zadoks $\sim$GS39). (B- D) Reverse transcription quantitative
PCR (RT-qPCR) analysis of the three β-diketone biosynthesis genes was performed in wheat BL glaucous and glossy internodes. Relative gene expression of (B) DMP, (C) DMH and (D) DMC, shown as means ±SD of n≥ 3 biological replicates, *** p<0.001. (E) Co-expression analysis, r-value cut-off (unless indicated otherwise in numbers and grey lines) = 0.85. Grey and yellow circles on dashed black and red lines show numbers of genes co-expressed with two or all three β-diketone biosynthesis genes, respectively. WES and CYP96B30 are among the genes that are co-expressed with the DMP, DMH and DMC genes.

**Figure 4.** Virus-induced gene silencing of DMP and DMH in wheat affects β-diketone biosynthesis and results in a glossy appearance. (A) Phenotypes in spikes and leaf sheaths of plants infected with different VIGS constructs: BSMV:mcs4D (control), BSMV:asDMP-1, BSMV:asDMP-2, BSMV:asDMH. (B, C) Relative expression levels of the targeted genes DMP (B) and DMH (C) in flag leaf sheath. (D, E) Analysis of β-diketones (D) and hydroxy-β-diketones (E) in flag leaf sheaths of plants infected with different VIGS constructs. Relative peak area = compound pick area/ internal standard peak area. (E). Values indicate the mean ±SD of n≥ 4 biological replicates. as, antisense. * p < 0.05, *** p < 0.005.

**Figure 5.** Barley cer mutants have mutations in β-diketone biosynthesis genes DMP, DMH, and DMC. (A) A screen of barley cer mutants revealed eight mutants with either premature termination codon (PTC) or deletions in the β-diketone genes DMP, DMH, and DMC. cer-q.56 and cer-q.82 have the same mutation in the DMH gene, hence it cannot be determined if these are two different mutants. (B- D) RT-qPCR analysis of the gene cluster in WT bonus and cer-cqu.724 triple mutant supports a deletion in the genetic region comprising DMP, DMH, and DMC. Relative expression is shown for (B) DMP, (C) DMH, and (D) DMC. Values indicate the mean ±SD of n≥ 3 biological replicates. ** p<0.005, *** p<0.001.

**Figure 6.** Heterologous expression of barley DMH suggests its biochemical activity and leads to a revised β-diketone biosynthesis pathway. (A) Lipid analysis of DMH-
expressing bacteria revealed two compounds not present in the control (C15 2-ketone and C15 2-alkanol). In addition, C14 3-hydroxy-acid increased in HvDMH-expressing cells. C15 2-ketone is a key intermediate most probably derived from decarboxylation (spontaneous upon GC-MS heating) of C16 3-ketoacid, the DMH reaction product. Values indicate the mean ±SD of n= 5 biological replicates. * p<0.05, *** p<0.005. Relative peak area = compound peak area / internal standard peak area. (B) Revised β-diketone biosynthesis pathway based on the evidence presented in the current study. Sequential arrows stand for possible sequential enzymatic reactions. Dashed arrows represent reactions that explain the E. coli findings, however it is not clear whether or not they also occur in planta. DMP, DMH, and DMC are shown in blue. WES and CYP96B30, which were found to be located within the cluster region in the wild emmer wheat Zavitan reference genome, are shown in yellow. Enzymes from the FAS system are shown in grey. Methylketones, 2-alkanols and 2-alkanols ester that require DMH are shown in light green. β-diketones and Hydroxy-β-diketones that require DMH, DMP and DMC (DMC only for Hydroxy-β-diketones) are shown in light pink. Alkylresorcinol predicted biosynthesis pathway is shown in light purple, based on von Wettstein-Knowles (2012). Spont. = spontaneous. a, n, m, x and y are placeholders to designate chain length variation.
De-novo assembly of the wheat transcriptome resulted in two different transcripts matching one gene in both wheat and barley. comp186033 matches the upstream part of the gene and comp166115 matches the downstream part. Together they cover the whole gene in both wheat and the barley homolog MLOC_13397.

Candidate genes based of Zavitan region analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Transcript # in wheat transcriptome data</th>
<th>Expression fold change BL/ CASL*2BS</th>
<th>Hits in wheat 2BS Chinese spring reference genome</th>
<th>Hits in wheat 2DS Chinese spring reference genome</th>
<th>Barley homologue in 2HS</th>
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<td>Traes_2DS_4ABDF6D9C</td>
<td>not found</td>
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</table>

** Candidate genes based of Zavitan region analysis.
Table 2. The wheat gene cluster (accession Zavitan) is conserved in the barley genome

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<tr>
<th>Zavitan gene(s)</th>
<th>Hit in the barley cluster region</th>
</tr>
</thead>
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<tr>
<td>NB-Arc-like_2</td>
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<tr>
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<tr>
<td>PKS-2 (2.1, 2.2)</td>
<td>MLOC_59804</td>
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<tr>
<td>PKS-3</td>
<td>Alignment to an un-annotated sequence within the cluster region</td>
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<tr>
<td>Ank_PGG</td>
<td>No alignment in the cluster region</td>
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</tbody>
</table>
**Figure 1.** Differences in wax phenotypes between the glaucous BL and the glossy CASL*2BS wheat lines. (A) BL is covered with bluish-grey, waxy material while CASL*2BS is glossy. (B) The epicuticular wax covering peduncle and flag leaf (abaxial side) tissues was examined using Cryo Scanning Electron Microscopy. BL and CASL*2BS lines exhibit different wax amounts and crystals shapes. Tubule-shaped crystals predominate on BL while crystals on CASL*2BS are mostly platelet-shaped. Magnification: 10 k. Scale bar indicates 2 µm. Chemical compositions of wheat BL and CASL*2BS wax mixtures on (C) flag leaf blade and (D) flag leaf sheath plus peduncle were determined by GC-MS analysis. Values indicate the mean ±SD of n≥ 4 biological replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. 
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A. Upper glaucous internode
Lower glossy internode

B. Bar graph showing relative transcript level of DMP.

C. Bar graph showing relative transcript level of DMH.

D. Bar graph showing relative transcript level of DMC.

E. Network diagram indicating the relationships between DMP, DMH, DMC, CYP, and WES, with numeric values indicating correlation coefficients (0.967, 0.966, 0.899, 0.966, 0.967).
**Figure 3.** The three $\beta$-diketone metabolic gene cluster members are highly expressed in glaucous tissues and have very similar expression patterns. (A) A wheat tiller in the stem elongation stage in which the upper internode is already glaucous while the next lower internode is still glossy (Zadoks $\sim$GS39). (B-D) Reverse transcription quantitative PCR (RT-qPCR) analysis of the three $\beta$-diketone biosynthesis genes was performed in wheat BL glaucous and glossy internodes. Relative gene expression of (B) DMP, (C) DMH and (D) DMC, shown as means ±SD of n≥ 3 biological replicates, *** $p<0.001$. (E) Co-expression analysis, $r$-value cut-off (unless indicated otherwise in numbers and grey lines) = 0.85. Grey and yellow circles on dashed black and red lines show numbers of genes co-expressed with two or all three $\beta$-diketone biosynthesis genes, respectively. *WES* and *CYP96B30* are among the genes that are co-expressed with the *DMP, DMH* and *DMC* genes.
A. Images showing the comparison of BSMV:mcs4D (control) with BSMV:asDMP-1, BSMV:asDMP-2, and BSMV:asDMH in Spike and Leaf sheath.

B. Graphs showing relative expression levels of DMP. BSMV:mcs4D, BSMV:asDMP-1, and BSMV:asDMP-2 are compared. BSMV:mcs4D shows the highest expression level, followed by BSMV:asDMP-1 and then BSMV:asDMP-2.

C. Graphs showing relative expression levels of DMH. BSMV:mcs4D shows the highest expression level, followed by BSMV:asDMH.

D. Graphs showing relative pick area of β-diketone. BSMV:mcs4D shows the highest pick area, followed by BSMV:asDMP-1, BSMV:asDMP-2, and BSMV:asDMH.

E. Graphs showing relative pick area of Hydroxy-β-diketone. BSMV:mcs4D shows the highest pick area, followed by BSMV:asDMP-1, BSMV:asDMP-2, and BSMV:asDMH.
**Figure 4.** Virus-induced gene silencing of *DMP* and *DMH* in wheat affects β-diketone biosynthesis and results in a glossy appearance. (A) Phenotypes in spikes and leaf sheaths of plants infected with different VIGS constructs: BSMV:mcs4D (control), BSMV:asDMP-1, BSMV:asDMP-2, BSMV:asDMH. (B, C) Relative expression levels of the targeted genes *DMP* (B) and *DMH* (C) in flag leaf sheath. (D, E) Analysis of β-diketones (D) and hydroxy-β-diketones (E) in flag leaf sheaths of plants infected with different VIGS constructs. Relative peak area = compound pick area/ internal standard peak area. (E). Values indicate the mean ±SD of n≥ 4 biological replicates. as, antisense. * p < 0.05, *** p < 0.005.
<table>
<thead>
<tr>
<th>Barley mutant</th>
<th>Cultivar</th>
<th>Mutagenesis method</th>
<th>Phenotype in internodes and spikes</th>
<th>Candidate gene in which a mutation was found</th>
<th>Mutation effect</th>
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<tbody>
<tr>
<td>cer-c.36</td>
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<td>glossy</td>
<td>DMP</td>
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<td>DMH+ DMC</td>
<td>putative deletion</td>
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<tr>
<td>cer-cqu.724</td>
<td>Bonus</td>
<td>neutrons</td>
<td>glossy</td>
<td>DMP+ DMH+ DMC</td>
<td>putative deletion</td>
</tr>
</tbody>
</table>

![Barley mutant expression levels](image)

**A**

**B**

**C**

**D**
Figure 5. Barley cer mutants have mutations in β-diketone biosynthesis genes DMP, DMH, and DMC. (A) A screen of barley cer mutants revealed eight mutants with either premature termination codon (PTC) or deletions in the β-diketone genes DMP, DMH, and DMC. cer-q.56 and cer-q.82 have the same mutation in the DMH gene, hence it cannot be determined if these are two different mutants. (B-D) RT-qPCR analysis of the gene cluster in WT bonus and cer-cqu.724 triple mutant supports a deletion in the genetic region comprising DMP, DMH, and DMC. Relative expression is shown for (B) DMP, (C) DMH, and (D) DMC. Values indicate the mean ±SD of n≥ 3 biological replicates. ** p<0.005, *** p<0.001
**A**

- Relative pick area
  - C14 3-OH
  - C15 2-ketone
  - C15 2-ol

**B**

- **Fatty acid metabolism (plastid)**
  - Acyl-ACP
  - KAS
  - 3-ketoacyl-ACP
  - KAR
  - 3-hydroxyacyl-ACP
  - HAD
  - 2,3-enoyl-ACP
  - ENR
  - Acyl-ACP
  - **Fatty acid metabolism (ER)**
  - Type III PKS
  - **Tetraketide intermediate**
  - **Alkylresorcinol**

- **β-diketone**
- **DMH**
- **DMP**
- **DMC (CYP709J4)**
- **Hydroxy-β-diketone**
- **CYP96B30**
- **2-ketone (methylketone)**
- **2-alkanol**
- **2-alkanol ester**

- **C14 3-OH**
- **C15 2-ketone**
- **C15 2-ol**

- Empty vector
- Hydrolase

***

- Spont.?
Figure 6. Heterologous expression of barley DMH suggests its biochemical activity and leads to a revised β-diketone biosynthesis pathway. (A) Lipid analysis of DMH-expressing bacteria revealed two compounds not present in the control (C_{15} 2-ketone and C_{15} 2-alkanol). In addition, C_{14} 3-hydroxy-acid increased in HvDMH-expressing cells. C_{15} 2-ketone is a key intermediate most probably derived from decarboxylation (spontaneous upon GC-MS heating) of C_{16} 3-ketoacid, the DMH reaction product. Values indicate the mean ± SD of n= 5 biological replicates. * p<0.05, *** p<0.005. Relative peak area = compound peak area / internal standard peak area. (B) Revised β-diketone biosynthesis pathway based on the evidence presented in the current study. Sequential arrows stand for possible sequential enzymatic reactions. Dashed arrows represent reactions that explain the E. coli findings, however it is not clear whether or not they also occur in planta. DMP, DMH, and DMC are shown in blue. WES and CYP96B30, which were found to be located within the cluster region in the wild emmer wheat Zavitan reference genome, are shown in yellow. Enzymes from the FAS system are shown in grey. Methylketones, 2-alkanols and 2-alkanols ester that require DMH are shown in light green. β-diketones and Hydroxy-β-diketones that require DMH, DMP and DMC (DMC only for Hydroxy-β-diketones) are shown in light pink. Alkylresorcinol predicted biosynthesis pathway is shown in light purple, based on von Wettstein-Knowles (2012). Spont. = spontaneous. a, n, m, x and y are placeholders to designate chain length variation.


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A Metabolic Gene Cluster in the Wheat W1 and the Barley Cer-cqu Loci Determines β-Diketone Biosynthesis and Glaucousness


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